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British Journal of Pharmacology

September 1977

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The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription UK £52.50; USA & Canada, US $115; Rest of world, US $105; Airmail, US $140 (or equivalent in any other currency). (Orders must be accompanied by remittance. Prices correct at time of publication.)

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Second class postage paid at New York, N.Y.
US Mailing Agent, Expeditors of the Printed Word, Ltd,
527 Madison Avenue, New York, NY 10022.

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HYPOTHALAMIC ADRENALINE SYNTHESIS AFTER STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE

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1 The problem of whether locally released noradrenaline can be methylated to adrenaline in the hypothalamus has been investigated.

2 During stimulation of the medial forebrain bundle (MFB) the hypothalamic adrenaline content increased somewhat, but the increase was not statistically significant (13%, mean of 10 experiments).

3 After inhibition of the activity of monoamine oxidase and catechol-*O*-methyltransferase this increase was much larger (80%, mean of 9 experiments).

4 Adrenalectomy did not prevent the rise in hypothalamic adrenaline after stimulation of the MFB. These results suggest that noradrenaline released during activity of noradrenergic hypothalamic structures may be methylated to adrenaline in the hypothalamus.

Introduction

This paper describes an investigation into whether the noradrenaline released by stimulation of the medial forebrain bundle (MFB) in the hypothalamus (Torda, 1977) is, at least in part, methylated locally to adrenaline.

Methods

Adult male cats were used. Two weeks before the experiment they were anaesthetized with halothane-nitrous oxide and a monopolar platinum microelectrode (0.5–1 μm tip diameter) was lowered into the medial forebrain bundle at A 10.3; L 2.1 and D −3.8 mm stereotaxic coordinates (Snider & Niemer, 1961). A chemitrode was implanted in addition into the ventromedial area of the hypothalamus to allow drugs to be injected. It consisted of a 14.5 mm long 28-gauge hypodermic needle inside a 14 mm long 22-gauge needle (Brown & Fial, 1975; Laroux & Myers, 1975). Electrodes and chemitrodes were fixed to the skull with acrylic cement. Tip positions were ascertained posthumously.

The cats were divided into 4 groups: (1) non-stimulated, non-drug-injected controls; (2) stimulated, non-drug-injected; (3) non-stimulated, drug-injected; (4) stimulated, drug-injected. The drugs injected into groups 3 and 4 were a combination of pyrogallol (4 mg/kg), an inhibitor of catechol-*O*-methyltransferase (COMT), and SU 11739 (Ciba, *N*-methyl-*N*-2-propynyl-1-indamine, 40 mg/kg), an inhibitor of monoamine oxidase (Maitre, 1967). The substances were dissolved in small amounts of 0.9% w/v NaCl solution (saline) and injected into the chemitrode with a Hamilton syringe 50 min before decapitation of the cats. The medial forebrain bundle (MFB) of the cats in groups 2 and 4 was stimulated 20 min before decapitation: rectangular pulses (0.2 ms, 40 Hz, 30–100 nA; 0.15 s pulse sets alternated with 1 s rest periods) were generated by means of a Tektronix Pulse Generator (Series 160, 161, 162). The current was delivered across a stimulus isolation unit to the implanted microelectrode. In order to simulate physiological conditions, stimulus intensities were kept to a minimum. These near-threshold current intensities were sufficient to activate noradrenergic processes, partially because of summation during the 20-min stimulation period.

Assay of noradrenaline and adrenaline

These substances were extracted together from the hypothalamus by the method of Braestrup, Nielsen & Scheel-Kruger (1974), and were assayed fluorimetrically following the method of Compuzano, Wilkerson & Horvath (1975). The threshold of the method was 500 times lower than the smallest amounts of adrenaline found in the hypothalamus.

Table 1 Effect of electrical stimulation of the medial forebrain bundle on the hypothalamic noradrenaline and adrenaline content

		Noradrenaline (NA)				*Adrenaline (A)*					
		Flourimetric method		*Radioenzymatic assay*		*Fluorimetric method*			*Radioenzymatic assay*		
	No. of cats	*(ng per g tissue)*	*(% of NA in control sample)*	*(ng per g tissue)*	*(% of NA in control sample)*	*(ng per g tissue)*	*(% of NA in same sample)*	*(% of A in control samples)*	*(ng per g tissue)*	*(% of NA in same sample)*	*(% of A in control samples)*
Controls	10	1317 ± 11.23	100	1264 ± 16.50	100	149 ± 4.04	11	100	164 ± 5.20	13	100
Stimulated	10	1198 ± 10.03	91	1138 ± 15 99	90	192 ± 5.53	16	128*	204 ± 6.24	18	124*
Cats injected with pyrogallol and SU 11739											
Controls	8	1386 ± 15.57	100			158 ± 5.93	12	100			
Stimulated	9	1296 ± 16.38	94			285 ± 5.58	22	180*			

Values are mean ± s.e. mean.

*Comparison with unstimulated controls ($P < 0.001$).

The adrenaline and noradrenaline contents of the samples were assayed simultaneously using 410 nm excitation and 505 nm emission wavelength for adrenaline and 395 nm excitation and 485 nm emission wavelength for noradrenaline. All estimations were made in duplicate. The methods of the two groups of authors were followed exactly and the precision of assays could be reproduced. Recoveries of adrenaline and noradrenaline from biological samples were estimated by adding standard solutions of catecholamines (100 ng/ml) containing tracer amounts of tritium labelled adrenaline (from 10 to 500 ng) and ^{14}C-labelled noradrenaline (100 to 1500 ng). Recoveries were in the order of 97%.

Hypothalamic adrenaline and noradrenaline content of cats in groups 1 and 2 (not injected with pyrogallol) were also assayed by the radioenzymatic method of Passon & Peuler (1973). Recoveries of added adrenaline and noradrenaline from tissue extracts reproduced the values obtained by Passon & Peuler with a maximum difference of 2%. As the radioenzymatic assay is based on the enzymatic ntroduction of an *O*-methyl group into the atecholamine molecule by the action of COMT, it could not be used for tissues from cats treated with the COMT inhibitor.

Materials

All materials were purchased from New England Nuclear Co., Calbiochem, and Fisher Scientific Co.

Adrenalectomy

One week before the acute experiments trans-abdominal adrenalectomy was performed on a group of cats. During the postoperative period the cats were given 5 mg/kg cortisone and 0.5 mg/kg aldosterone daily.

Statistical evaluation

The statistical significance of the observed differences between mean values was tested by Student's *t*-test.

Results

In control cats the hypothalamic noradrenaline content average 1317 ng per g and the adrenaline content 149 ng per g wet weight. Stimulation of the MFB caused an increase in the hypothalamic adrenaline concentration by 24 or 28% ($P < 0.001$) depending on the assay (Table 1). In cats in which the catabolism of noradrenaline and adrenaline by monoamine oxidase and COMT was prevented by the injection of pyrogallol and SU 11739, electrical stimulation of the MFB caused a considerably larger rise (± 80%) in hypothalamic adrenaline than in non-injected cats (see Table 1). In both groups there was a slight but not significant fall in the noradrenaline concentrations. That the rise in the adrenaline content of the hypothalamus after MFB stimulation in the drug-treated cats was not due to uptake from the blood stream of adrenaline released from the adrenal medulla can be inferred from the results obtained on adrenalectomized cats (see Table 2). Adrenalectomy was without effect on the adrenaline concentrations in the hypothalami of the control cats. However, there was still a rise of 78% in the hypothalamic adrenaline concentrations after stimulation of the MFB in those cats which were injected with the enzyme inhibitors.

Discussion

Adrenaline, catecholaminergic neurones and specific phenylethanolamine-*N*-methyltransferases have been located in the same brain regions. The principal

Table 2 Intrahypothalamic synthesis of adrenaline during stimulation of the medial forebrain bundle in adrenalectomized cats

	No. of cats	*Adrenaline content of hypothalamus* *(ng per g tissue)*	*(% of controls)*
Controls	5	131 ± 11.3	100
Stimulated	5	160 ± 12.5	122
Cats injected with pyrogallol and SU 11739			
Controls	4	139 ± 12.2	100
Stimulated	4	247 ± 13.4*	178*

Values are mean ± s.e. mean.

*Comparison with unstimulated controls ($P < 0.005$).

source of intracerebral adrenaline is methylation of noradrenaline by one of the specific phenylethanolamine-*N*-methyltransferases (Axelrod, 1968; Ciaranello, Barchas, Byers, Stemmle & Barchas, 1969; Pohoreczky, Zigmond, Karlen & Wurtman, 1969; Joh & Goldstein, 1973; Eagles & Iqbal, 1974; Lee, Schultz & Fuller, 1974; Laduron, 1974; Pendleton & Gessner, 1975; Saavedra, Grobecker & Axelrod, 1976; Torda, 1976a, b).

Electrical stimulation of the MFB releases hypothalamic noradrenaline in the cat (Torda, 1977). This noradrenaline is either removed by the blood stream, or by reuptake processes, or it is catabolized (in addition to combining with postsynaptic receptors). In the present work evidence was obtained that part of the noradrenaline released was methylated to adrenaline (Table 1). Inhibition of the two principle enzymes involved in catecholamine catabolism, monoamine oxidase and COMT, increased the rise in hypothalamic adrenaline after stimulation of the MFB from 28 to 80%.

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(Received July 12, 1976.
Revised March 21, 1977.)

Br. J. Pharmac. (1977), **61**, 9–17

ASSESSMENT OF THE SELECTIVITY OF OPC-2009, A NEW β_2-ADRENOCEPTOR STIMULANT, BY THE USE OF THE BLOOD-PERFUSED TRACHEA *in situ* AND OF THE ISOLATED BLOOD-PERFUSED PAPILLARY MUSCLE OF THE DOG

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1 The potency and selectivity of 5-(1-hydroxy-2-isopropylamino)butyl-8-hydroxy carbostyril hydrochloride hemihydrate (OPC-2009), a new β_2-adrenoceptor stimulant, was compared with those of isoprenaline, trimetoquinol and salbutamol by the use of blood-perfused tracheal preparations *in situ* and of blood-perfused papillary muscle preparations of the dog. All drugs were injected intra-arterially.

2 All the four drugs decreased tracheal intraluminal pressure (tracheal relaxation) and increased tracheal blood flow in a dose-dependent manner. The four drugs produced a dose-dependent increase in developed tension of papillary muscles. In both preparations the duration of action of isoprenaline and salbutamol was short, whereas that of OPC-2009 and trimetoquinol was long. These effects were antagonized by propranolol.

3 Dose-response curves to the four drugs for tracheal relaxation were almost parallel. OPC-2009 was 2.4 times more potent, and trimetoquinol and salbutamol were 2.2 and 6.2 times less potent than isoprenaline in causing tracheal relaxation.

4 Dose-response curves to the four drugs for tracheal vasodilatation were also parallel. OPC-2009, trimetoquinol and salbutamol were 3.9, 6.7 and 23 times less potent than isoprenaline.

5 Slopes of the dose-response curves to the four drugs for increased developed tension were not parallel; that of OPC-2009 was the least steep, whereas that of isoprenaline was the steepest. Trimetoquinol, salbutamol and OPC-2009 were about 18, 570 and 2400 times less potent than isoprenaline.

6 Selectivity calculated from relative potencies indicate that OPC-2009 was about 6000 times, salbutamol about 92 times and trimetoquinol about 8.2 times more selective than isoprenaline for tracheal smooth muscle as compared to ventricular muscle.

7 The high potency and selectivity of OPC-2009 for tracheal smooth muscle and its long duration of action suggest its potential usefulness for treatment of bronchial asthma.

8 The present results are also compatible with the concept that β_1-adrenoceptors in cardiac muscle and β_2-adrenoceptors in tracheal and vascular smooth muscle can be distinguished. Furthermore, the results revealed that the β-adrenoceptors mediating tracheal relaxation and vasodilatation may also be different.

Introduction

A β-adrenoceptor stimulant, isoprenaline is one of the most popular bronchodilators but has certain disadvantages. They consist firstly of a rather short duration of action owing to tissue uptake (Gryglewski & Vane, 1970), rapid biotransformation to inactive metabolites (Ross, 1963; Conway, Minatoya, Lands & Shekosky, 1968), or both (Morgan, Sandler, Davies, Conolly, Paterson & Dollery, 1969), and secondly of marked side effects due to its action on cardiovascular β-adrenoceptors. This has stimulated search for bronchodilators that are more selective for bronchial than for cardiovascular β-adrenoceptors and longer lasting in their action. Trimetoquinol (Iwasawa & Kiyomoto, 1967; Sato, Yamaguchi & Kiyomoto, 1967), soterenol (Dungan, Cho, Gomoll, Aviado & Lish, 1968), salbutamol (Hartley, Jack, Lunts &

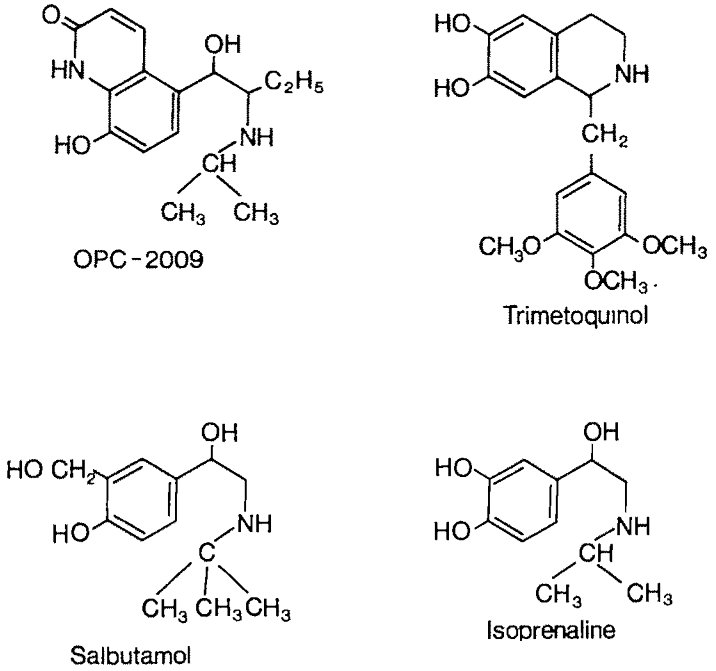

Figure 1 Structures of 5-(1-hydroxy-2-isopropylamino)butyl-8-hydroxy carbostyril (OPC-2009), trimetoquinol, salbutamol and isoprenaline.

Ritchie, 1968; Brittain, Farmer, Jack, Martin & Simpson, 1968; Cullum, Farmer, Jack & Levy, 1969), rimiterol (Bowman & Rodger, 1972), Th1165a (Giles, Williams & Finkel, 1973) and carbutenol (Wardell, Colella, Shetzline & Fowler, 1974) have been developed as such drugs. Lands, Arnold, McAuliff, Luduena & Brown (1967a) and Lands, Luduena & Buzzo (1967b) have proposed that β-adrenoceptors may be pharmacologically differentiated into β_1-adrenoceptors in cardiac muscle and β_2-adrenoceptors in bronchial and vascular smooth muscle. Thus, the broncho-dilators listed above can be classified as selective β_2-adrenoceptor stimulants. Recently, Yoshizaki, Tanimura, Tamada, Yabuuchi & Nakagawa (1976) synthesized a powerful bronchodilator having a β-adrenoceptor stimulant action, 5-(1-hydroxy-2-isopropylamino)butyl-8-hydroxy carbostyril hydrochloride hemihydrate, OPC-2009 (Figure 1). OPC-2009 appears highly selective for β_2-adrenoceptors so far as its selectivity for β_2- *v* β_1-adrenoceptors is assessed in guinea-pig isolated tracheae and atria (Yoshizaka *et al.*, 1976). In general, however, the selectivities of β_2-adrenoceptor stimulants for β_2- *v* β_1-adrenoceptors depend upon animal species used for assessment and upon experimental procedures (Brittain, Jack & Ritchie, 1970).

In view of these, we attempted the present experiments to assess the potency and selectivity of OPC-2009 for β_2- *v* β_1-adrenoceptors in dog tracheal and vascular smooth muscle and ventricular muscle. For this purpose, we used the blood-perfused dog trachea *in situ* (Himori & Taira, 1975; 1976) which allows the injection of drugs selectively to the tracheal vascular bed and the simultaneous observation of responses of vascular and tracheal smooth muscle. We also used the blood-perfused papillary muscle preparation of the dog (Endoh & Hashimoto, 1970). Isoprenaline was used as a reference β-adrenoceptor stimulant in all experiments, and trimetoquinol and salbutamol were included for comparison.

Methods

Blood-perfused tracheal preparations in situ

Fourteen mongrel dogs of either sex, weighing 12–16.5 kg were anaesthetized with pentobarbitone sodium initially at a dose of 30 mg/kg, intravenously and during the experiment, anaesthesia was maintained by continuous infusion of the same anaesthetic at a rate of 5 mg kg^{-1} h^{-1}. The upper cervical region was incised in the midline, and the left and right superior thyroid arteries were dissected out. Muscular, pharyngeal and cricothyroid branches which derive from the superior thyroid arteries but do not supply the trachea were all ligated. After the animal had been given heparin sodium at a dose of 500 units/kg, intravenously, two arms of a Y-shape cannula were inserted into the left and right superior thyroid arteries. Arterial blood led from the right femoral artery was directed to the Y-shape cannula by the use of a peristaltic pump (Harvard Apparatus, Model 1215). Constant pressure perfusion was accomplished by shunting a fraction of the blood to the femoral vein through a Starling pneumatic resistance set in parallel with the perfusion circuit. The perfusion pressure was monitored at a side arm of the perfusion circuit and the systemic blood pressure was measured at the femoral artery by an individual pressure transducer (Nihon Kohden, MPU-0.5). The perfusion pressure was adjusted initially to give a value approximating the mean systemic blood pressure and kept constant throughout the experiment. The blood flow through the perfused arteries was measured with an electromagnetic flow meter (Nihon Kohden, MF-46-3) set just proximal to the Y-shape cannula. During the experiment heparin sodium (100 units/kg) was given at hourly intervals.

Responses of tracheal smooth muscle were measured as changes in the intraluminal pressure of a water-filled cuff (5 cm in length) of a tracheal tube introduced into the trachea via the mouth. The water-filled cuff was located from 3 to 8 cm caudal to the larynx and connected to a pressure transducer (Nihon Kohden, LPU-0.1) through polyethylene tubing. The resting intraluminal pressure was adjusted to 25–35 cmH_2O. Details of the preparation have been given by Himori & Taira (1976).

Blood-perfused papillary muscle preparation

Nine preparations of the anterior papillary muscle of the right ventricle of the heart were obtained from mongrel dogs of either sex, weighing 9.5–14 kilograms. The preparations were placed in a water jacket warmed at 38–39°C and perfused through the cannulated anterior septal artery at a constant pressure of about 100 mmHg with blood from the right carotid artery of a donor dog. Constant pressure perfusion was achieved by the use of a peristaltic pump (Harvard Apparatus, Model 1215) and a Starling pneumatic resistance set in parallel with the preparation. The venous blood from the preparation and blood passing through the pneumatic resistance was collected by a funnel and returned to the donor dog through the right external jugular vein. The papillary muscle preparations were driven with square-wave pulses of about twice the threshold voltage (0.6–1.4 V) and 5 ms duration at a rate of 2 Hz delivered by an electronic stimulator (Nihon Kohden, MSE 3) via bipolar silver electrodes sutured near the base of the papillary muscle. Isometric contractions of the papillary muscle loaded with a weight of about 1.5 g, were picked up with a force-displacement transducer (Grass, FTO3B) and recorded on a recticorder (San-ei Instrument, Rectiholiz 8S). The blood flow through the anterior septal artery was monitored with an electromagnetic flow meter (Nihon Kohden, MF-46-3).

Donor dogs were anaesthetized initially with pentobarbitone sodium, 30 mg/kg, intravenously and with supplemental doses of about 4.5 mg $kg^{-1} h^{-1}$, intravenously. Heparin sodium, 500 units/kg, intravenously was given initially and 100 units/kg, intravenously was added at hourly intervals. Details of this preparation have been given by Endoh & Hashimoto (1970).

Drugs used in this study were (−)-isoprenaline hydrochloride (Nikken Kagaku), (±)-OPC-2009 (Otsuka), (±)-propranolol hydrochloride (ICI), (±)-salbutamol sulphate (Leiras) and (±)-trimetoquinol hydrochloride (Tanabe). All these drugs were dissolved in 0.9% w/v NaCl solution (saline) and diluted to desired concentrations with saline. Drug solutions in a volume of 30 µl (in 4 s) were injected by the use of microsyringes into rubber tubing just proximal to the cannulated arteries. Doses of drugs used refer to their bases. Two to four kinds of drugs were administered to each preparation in which the order of drug administration was randomized.

Values in the text are arithmetic means ± s.e. (unless otherwise stated). Differences in mean ED values between agonists were analysed by Student's t test and taken to be significant when $P < 0.05$. Parallelism of dose-response curves was analysed by the use of analysis of covariance techniques described by Snedecor & Cochran (1967). The dose-response curves were treated as linear regressions and analysed for similarities in slope. The criterion for significance was $P < 0.05$.

Results

Blood-perfused tracheal preparations in situ

The average blood flow through the tracheal vascular bed of the 14 dogs was 9.2 ± 0.7 ml/min at the average perfusion pressure of 135 ± 4.7 (s.d.) mmHg. The average resting intraluminal pressure of the trachea was 29 ± 2.1 cmH_2O ($n = 14$).

Single intra-arterial injections of isoprenaline (3 ng–0.3 µg) produced a decrease in intraluminal pressure of the trachea, viz., tracheal relaxation and an increase in blood flow through the tracheal vascular bed, that is, vasodilatation in a dose-dependent manner. Figure 2 (top panel) shows one such experiment. Both tracheal and vascular responses to isoprenaline were reproducible and the preparations were sensitive enough to respond to intra-arterial doses of isoprenaline as small as 1–3 ng. Single intra-arterial injections of OPC-2009 (3 ng–0.1 µg), trimetoquinol (0.01–0.3 µg) and

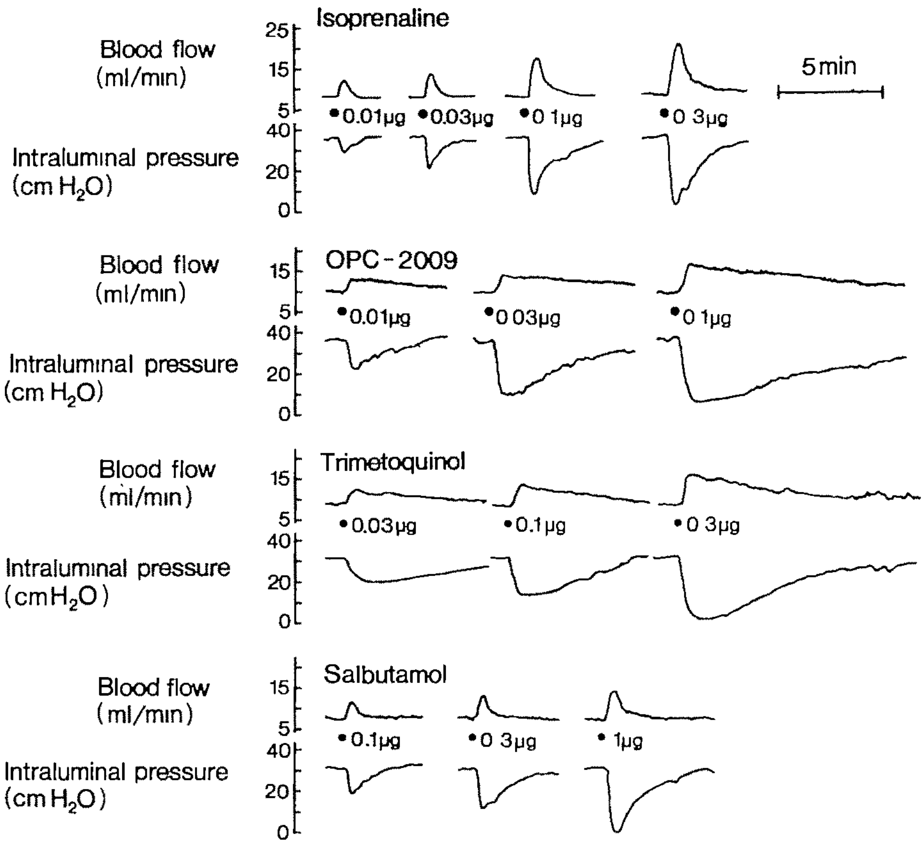

Figure 2 Responses of the tracheal vascular bed (blood flow) and musculature (intraluminal pressure) to intra-arterial isoprenaline, OPC-2009, trimetoquinol and salbutamol.

salbutamol (0.03–1 μg) also produced tracheal relaxation and vasodilatation in a dose-dependent manner (Figure 2). Dose-response curves to four compounds for tracheal relaxation and vasodilatation are shown in Figure 3. The dose-response curves to OPC-2009, trimetoquinol and salbutamol for tracheal relaxation were almost parallel to that of isoprenaline; slopes of these curves were not significantly different (Table 1). The same was true for the dose-response curves for tracheal vasodilatation. When compared in doses reducing the intraluminal pressure by 15 cmH_2O, ($ED_{15\,cmH_2O}$), OPC-2009 was 2·4 times more potent, and trimetoquinol and salbutamol were 2.2 and 6.2 times less potent than isoprenaline in producing tracheal relaxation (Table 2). When compared in doses increasing blood flow by 5 ml/min ($ED_{5\,ml/min}$), OPC-2009, trimetoquinol and salbutamol were 3.9, 6.7 and 23 times less potent than isoprenaline in causing tracheal vasodilatation (Table 2). As can be clearly seen in Figure 2, both tracheal relaxant and vasodilator effects of intra-arterial isoprenaline and salbutamol were brief, whereas those of intra-arterial OPC-2009 and trimetoquinol lasted longer. The tracheal relaxant and vasodilator effects of the four drugs were all antagonized by propranolol (60 μg, i.a.).

Blood-perfused papillary muscle preparations

The basal developed tension of the nine papillary muscles was 5.1 ± 0.3 g and the basal blood flow through the anterior septal artery was 6.2 ± 0.6 ml/minute.

Single intra-arterial injections of isoprenaline (300 pg–0.01 μg), trimetoquinol (3 ng–0.3 μg), salbutamol (0.3–10 μg) and OPC-2009 (0.1–10 μg) all produced a dose-dependent increase in developed tension, i.e., a positive inotropic effect. Typical experiments are shown in Figure 4 and dose-response curves for peak increase in developed tension are shown in Figure 5. As can be seen in Table 1 and

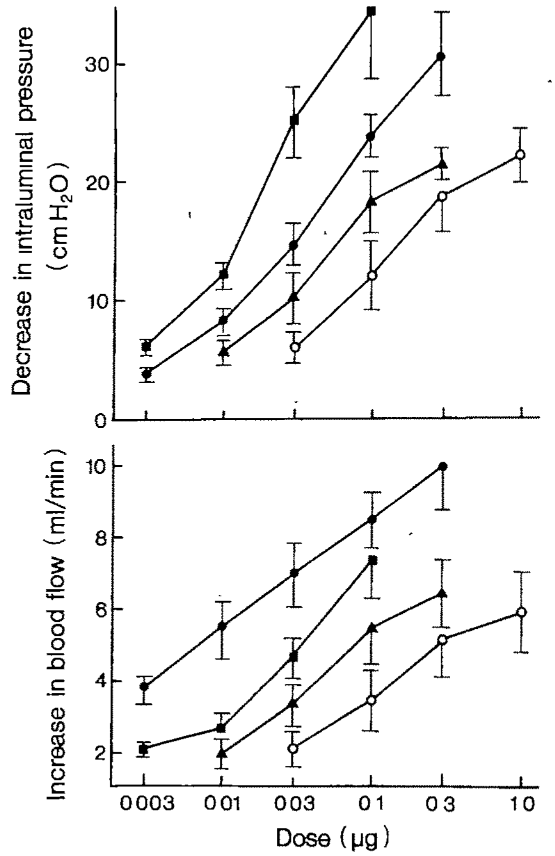

Figure 3 Dose-response curves for decrease in intraluminal pressure of and increase in blood flow through the trachea to intra-arterial isoprenaline (●), OPC-2009 (■), trimetoquinol (▲) and salbutamol (○). Each point represents the mean of 7–13 observations on 14 animals. Vertical bars show s.e. mean.

Figure 5 the slope of the dose-response curves for OPC-2009 was less steep than those for isoprenaline, trimetoquinol and salbutamol. Even with increasing doses of OPC-2009 above 10 μg, the effect of OPC-2009 did not appear to attain that of 0.01 μg of isoprenaline. When compared in doses increasing the developed tension by 2g (ED_{2g}, about 40% of the basal developed tension), trimetoquinol, salbutamol and OPC-2009 were approx. 18, 570 and 2400 times less potent than isoprenaline in increasing force of contraction of ventricular muscle (Table 2). Like the effects on the blood-perfused tracheal preparation, the positive inotropic effects of isoprenaline and salbutamol had a briefer duration of action than those of trimetoquinol and OPC-2009. The positive inotropic effects of the four drugs were antagonized by propranolol (3 μg, i.a.).

Table 1 Slopes of the dose-response curves shown in Figures 3 and 5

Tracheal smooth muscle	
	Slope
Isoprenaline (Iso)	15.5
OPC-2009 (OPC)	20.3
Trimetoquinol (Tri)	11.5
Salbutamol (Sal)	11.3
OPC/Iso	$F_{1,55}=1.87$
Tri/Iso	$F_{1,56}=1.51$
Sal/Iso	$F_{1,56}=1.34$
Tracheal vasculature	
	Slope
Iso	2.7
OPC	3.9
Tri	3.2
Sal	2.6
OPC/Iso	$F_{1,53}=0.26$
Tri/Iso	$F_{1,61}=0.11$
Sal/Iso	$F_{1,61}=0.01$
Papillary muscle	
	Slope
Iso	3.9
Tri	2.4
Sal	3.2
OPC	2.4
OPC/Iso	not calculated because non-parallelism is so evident
Sal/Iso	$F_{1,48}=0.59$
Tri/Iso	$F_{1,44}=4.95$

Selectivity for β-adrenoceptors of tracheal smooth muscle v *those of cardiac muscle*

As a measure of selectivity of the four drugs for β-adrenoceptors of tracheal smooth muscle *v* those of cardiac muscle, a ratio of ED_{2g} (the dose increasing the developed tension of papillary muscle by 2g) to $ED_{15\,cmH_2O}$ (the dose reducing the intraluminal pressure of the trachea by 15 cmH_2O) is calculated for each drug, being expressed as a value relative to that of isoprenaline which was taken as 1. The larger the value, the more selective is the action on β-adrenoceptors of tracheal smooth muscle. As shown in Table 2, the values were in descending order: OPC-2009 ≫ salbutamol > trimetoquinol > isoprenaline, being approximately 6000, 92, 8.2 and 1.

Discussion

In the present experiments tracheal relaxation and vasodilatation, and an increase in developed tension of papillary muscles in response to OPC-2009 were all antagonized by suitable doses of propranolol as were those to isoprenaline, salbutamol and trimetoquinol. Thus, OPC-2009 is a β-adrenoceptor stimulant and

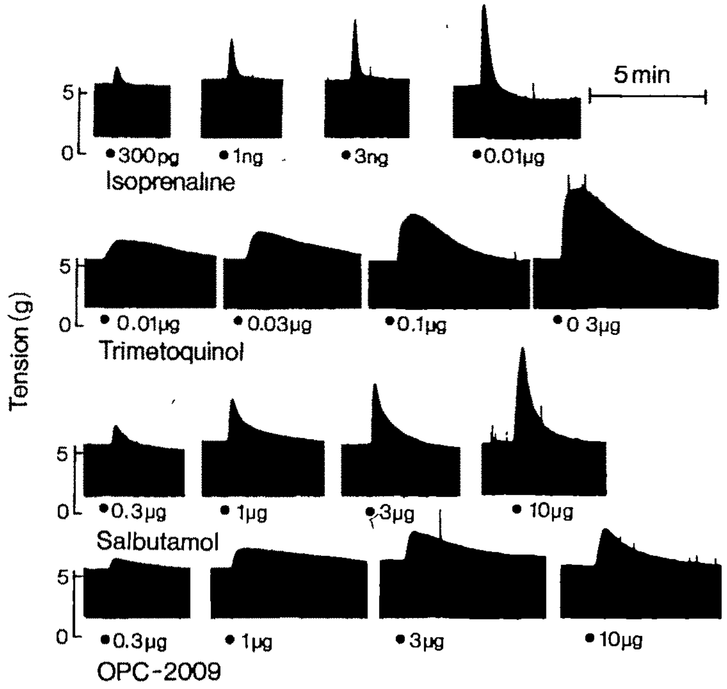

Figure 4 Effects of isoprenaline, trimetoquinol, salbutamol and OPC-2009 injected into the anterior septal artery on the developed tension of blood-perfused dog papillary muscles.

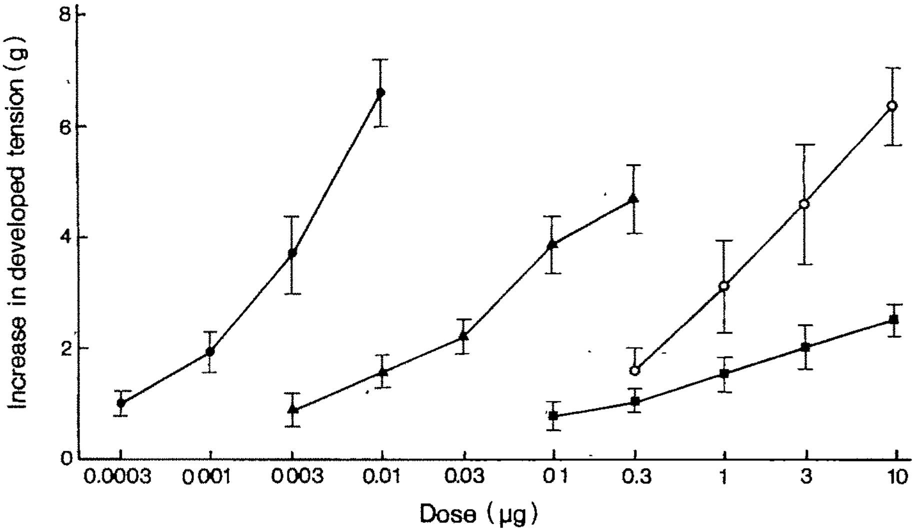

Figure 5 Dose-response curves for increase in developed tension of dog papillary muscles to intra-arterial isoprenaline (●), trimetoquinol (▲), salbutamol (○) and OPC-2009 (■). Each point represents the mean of 6–8 observations on 9 animals. Vertical bars show s.e. mean.

Table 2 Potencies and selectivity of isoprenaline, OPC-2009, trimetoquinol and salbutamol for β-adrenoceptors as determined in blood-perfused dog tracheal and papillary muscle preparations

	Tracheal				*Papillary*		
β-Adrenoceptor stimulants	*Musculature $ED_{15\,cmH_2O}$ (µg)*	*Relative potency*	*Vasculature $ED_{5\,ml/min}$ (µg)*	*Relative potency*	*muscle ED_{2g} (µg)*	*Relative potency*	*Selectivity for tracheal musculature*
Isoprenaline	0.037 ± 0.006 (13)	1	0.018 ± 0.006 (13)	1	0.0014 ± 0.0003 (8)	1	1
OPC-2009	0.015 ± 0.002* (8)	0.4	0.07 ± 0.03* (8)	3.9	3.4 ± 1.0* (7)	2400	6000
Trimetoquinol	0.08 ± 0.02* (8)	2.2	0.12 ± 0.03* (8)	6.7	0.025 ± 0.005* (6)	18	8.2
Salbutamol	0.23 ± 0.08* (7)	6.2	0.41 ± 0.20* (7)	23	0.8 ± 0.2* (7)	570	92

Doses are means ± s.e. mean. Number of preparations is shown in parentheses.

$ED_{15\,cmH_2O}$: Dose producing a decrease in intraluminal pressure of 15 cmH_2O. $ED_{5\,ml/min}$: Dose producing an increase in blood flow of 5 ml/minute. ED_2 g: Dose producing an increase in developed tension of 2 grams. Selectivity for tracheal musculature is calculated by the formula, $ED_{2g}/ED_{15\,cmH_2O}$ and expressed as a value relative to that of isoprenaline which is taken as 1.

* Significantly different from corresponding ED values of isoprenaline.

this is consistent with the conclusion drawn by Yoshizaki *et al.* (1976) for its action in guinea-pig tracheae and atria. In blood-perfused tracheal preparations the dose-response curves to OPC-2009, trimetoquinol and salbutamol for tracheal relaxation were almost parallel to that for isoprenaline. When the potencies of OPC-2009, trimetoquinol and salbutamol relative to that of isoprenaline were compared on the basis of doses decreasing the tracheal intraluminal pressure by 15 cmH_2O ($ED_{15\,cmH_2O}$), OPC-2009 was 2.4 times more potent and trimetoquinol and salbutamol were 2.2 and 6.2 times less potent than isoprenaline in causing tracheal relaxation (Table 2). The values for salbutamol and for trimetoquinol in the present experiments are in good agreement with those obtained previously in the ginea-pig isolated trachea (Brittain *et al.*, 1968; Cullum *et al.*, 1969; Farmer, Kennedy, Levy & Marshall, 1970). Thus the use of the present preparations for assessment of potencies of β-adrenoceptor stimulant bronchodilators is valid. It is worth noting that in the present preparations OPC-2009 was about 2.4 times as potent as isoprenaline in causing tracheal relaxation (Table 2).

In blood-perfused papillary muscle preparations the dose-response curves to the four drugs for increase in developed tension were not parallel. The slopes of the curves for salbutamol, trimetoquinol and OPC-2009 were less steep than that for isoprenaline in that order. Thus, it is not easy to determine their positive inotropic potencies relative to that of isoprenaline. Based on doses producing an increase in developed tension by 2 g (ED_{2g}) trimetoquinol, salbutamol and OPC-2009 were approx. 18, 570 and 2400 times less potent than isoprenaline in producing the positive inotropic effect on ventricular muscle of the dog heart. The value obtained for trimetoquinol is roughly equal to that determined on force of contraction of the heart *in situ* of open-chest dogs (Sato *et al.*, 1967). However, the value obtained for salbutamol does not agree with those reported in guinea-pig isolated left atria (Cullum *et al.*, 1969; Farmer *et al.*, 1970; Brittain *et al.*, 1970). The discrepancy between the values for salbutamol may be due to differences in animal species used, heart muscle (atrial or ventricular), experimental conditions (*in vitro* and *in vivo*), and the way in which dose-response curves were constructed. Indeed, in the present experiments no attempt has been made to obtain the maximum contractile response, because large doses tended to produce arrhythmias and a decrease in developed tension after an increase which resulted in deterioration of the preparations. It is noteworthy that OPC-2009 was about 2400 times less potent than isoprenaline in producing the positive inotropic effect on ventricular muscle of the dog heart.

Selectivity for β-adrenoceptors in tracheal smooth muscle as compared with those in ventricular muscle calculated from $ED_{15\,cmH_2O}$ and ED_{2g} was 6000 for OPC-2009, 92 for salbutamol and 8.2 for trimetoquinol. The high selectivity of OPC-2009 for β-adrenoceptors in tracheal smooth muscle taken together with its high potency in this preparation and its long duration of action suggests its potential usefulness for treatment of bronchial asthma. The present results showing a difference in selectivity for β-adrenoceptors in tracheal smooth muscle and ventricular muscle are also consistent with the proposal put forward by Lands *et al.* (1967a, b) that at least two types of β-adrenoceptors can be distinguished; β_1-type in cardiac muscle and β_2-type in bronchial and vascular smooth muscle.

In the tracheal vascular bed the dose-response curves to the four drugs for vasodilatation were almost parallel, and OPC-2009, trimetoquinol and salbutamol were 3.9, 6.7 and 23 times less potent than isoprenaline in causing tracheal vasodilatation. Thus, the relative potencies of the four drugs in producing tracheal vasodilatation are different from those in producing tracheal relaxation in which OPC-2009 was 2.4 times more potent and trimetoquinol and salbutamol were 2.2 and 6.2 times less potent than isoprenaline. OPC-2009 was 9.4 times, salbutamol 3.7 times and trimetoquinol 3.1 times more selective for tracheal smooth muscle than isoprenaline as compared to vascular smooth muscle. This indicates that OPC-2009 would have less hypotensive side effects than the other drugs. However, it would still be premature to draw such a conclusion since at present no information is available as to whether β-adrenoceptors in the whole vascular bed are homogeneous or not. The present results indicate that β-adrenoceptors mediating tracheal relaxation are different from those mediating vasodilatation in the dog trachea, as already suggested by previous workers (Bristow, Sherrod & Green, 1970; Wasserman & Levy, 1974, Wardell *et al.*, 1974) in various mammalian species.

We are grateful to Otsuka Pharmaceutical Co., for their generous supply of OPC-2009 and to Tanabe Pharmaceutical Co., for trimetoquinol.

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(Received August 16, 1976
Revised February 24, 1977.)

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INTERDEPENDENCE OF ION TRANSPORT AND THE ACTION OF OUABAIN IN HEART MUSCLE

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1 The influence of ouabain (0.4 μM) on contractile force and cellular Na and K concentrations was investigated in isolated left atria of the guinea-pig at rest and at different beat frequencies. Simultaneously the binding of ouabain to the tissue was determined.

2 Strict dependence of rates of onset of positive iontropic action and of binding of ouabain on beat frequency are limited to conditions where no alterations of cellular Na and K concentrations occur. A correlation was observed between sodium flux per unit time and the development of positive inotropism and binding to the receptors of ouabain.

3 Ouabain exerts its positive inotropic effect without affecting the intracellular Na and K concentrations in spite of the fact that under these conditions even the majority of binding sites, i.e. Na-K-adenosine triphosphatases (Na-K-ATPases), are occupied by the drug. The positive inotropic effect may be explained by a ouabain-induced conformational alteration of the Na-K-ATPase which leads to structural alterations of the plasmalemma connected with an increased availability of coupling calcium.

4 Increasing the frequency of stimulation over a critical value, which appears to be determined by an overloading of the Na pump, induces a decrease in contractile force, cellular accumulation of Na and loss of K, and eventually contracture.

5 The rate of binding of ouabain appears to depend on the actual concentration of particular conformations of the Na-K-ATPase with high affinity for ouabain. These conformations transiently occur during a pumping cycle and their concentration may therefore be dependent on the frequency of cycling which in turn is determined by the frequency of contraction.

6 Ouabain can easily be washed out from the tissue irrespective of the condition of the muscle. If, however, the intracellular Na and K homeostasis is impaired, the inhibition of the pump persists even if ouabain is released from the binding sites upon wash-out. It is suggested that the inhibition of the pump is maintained by an increased intracellular Ca ion concentration and a depletion of ATP.

7 A kinetic model is proposed for the interaction between cardiac glycosides and the Na-K-ATPase in intact heart muscle cells.

Introduction

The dependence of the actions of cardiac glycosides on frequency of contraction of heart muscle is still a matter of discussion. The dependence of rate of onset of cardiac glycoside-induced intoxication upon beat frequency is well known and repeatedly documented (Weizsäcker, 1913; Holland & Sekul, 1961). Weizsäcker (1913) stated that the number of contractions rather than the time of incubation determine the onset of toxic effects after addition of ouabain in frog ventricular muscle. However, cardiac glycosides are not completely inactive in the resting heart and will produce contracture (Weizsäcker, 1913). Yet, contractile activity considerably enhances the onset of toxic signs. While the majority of investigators agree on a beat-dependent onset of toxicity (Weizsäcker, 1913; Holland & Sekul, 1961; Gersmeyer & Holland, 1963) conflicting results are obtained with respect to a frequency-dependent rate of development of the cardiac glycoside-induced positive inotropism.

In general, at bath temperatures below 35°C, heart muscles of different species develop increased contractile force upon administration of cardiac glycosides in a strictly beat-dependent way (Wilbrandt, Brawand & Witt, 1953; Sanyal & Saunders, 1958; Gersmeyer & Holland, 1963; Holland, 1964; Moran, 1963; 1967; Byrne & Dresel, 1969). At a bath temperature of 35°C there still exists some beat-dependence at the higher frequencies of contraction and an obvious dependence upon time of incubation at lower frequencies (Vinzenci, 1967). If heart muscle is incubated at temperatures above 35°C the development of the positive inotropic effect becomes mainly time-dependent, though a certain influence of frequency remains involved (Garb & Penna, 1957; Koch-Weser & Blinks, 1962; Koch-Weser, 1962; 1971).

One of the basic actions of cardiac glycosides is to shorten the duration of the action potential concomitantly with the induction of enhanced contractile force. Recently it was demonstrated by Brinkmann & Ravens (1976) that shortening of the action potential below a critical duration will counteract the development of the positive inotropic effect. If both actions of cardiac glycosides, shortening of the action potential duration and positive inotropism, are initiated via one common mechanism, then the time course of development of the positive inotropic effect may be altered by the concomitantly occurring electrophysiological changes. Calculations of the respective half lives may be false and a correlation between frequency of contraction and time course of development of positive inotropism may be obscured. Both high temperature and high beat frequency *per se* tend to shorten the duration of the action potential (Shanes, 1958), and upon addition of cardiac glycosides the critical shortening will be attained earlier. In consequence one may speculate that it is only at low temperatures and within a limited range of beat frequency that the development of positive inotropism can be correlated to the frequency of contraction. From the foregoing it is understandable that several investigators sought to gain more insight into the relation between beat frequency and molecular mode of action of cardiac glycosides by means of binding studies which can be considered to be a more direct approach (Weizsäcker, 1913; Roth-Schechter, Anderson & Richardson, 1970; Deutscher, Harrison & Goldman, 1972). Unfortunately the experiments were carried out only with hydrophobic cardiac glycosides which are predominantly taken up by cardiac tissue in a nonspecific way, i.e. time course of uptake gives no information on the time course of binding of the drugs to a specific saturable compartment which may be linked to the inotropic effect (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967; Lüllmann, Peters & Ravens, 1975a). Accordingly, no correlation between uptake and frequency of contraction could be observed.

For the reasons outlined above, the present investigation was carried out at a comparatively low temperature (32°C) and with ouabain which, at the concentration applied, is taken up nonspecifically by heart muscle to a minor extent, the major amount being bound specifically to a saturable binding compartment (Godfraind & Lesne, 1972; Lüllmann *et al.*, 1975a). The purpose of the study was to gain further information concerning (1) the influence of frequency of contraction on the binding rate of ouabain, (2) the possible correlation between binding and the positive inotropic action of ouabain, and (3) the interdependence between transmembrane excitation sodium fluxes, Na- and K-transport, adenosine triphosphatase (ATPase)-activity and the processes of binding and positive inotropic action of ouabain.

In order to investigate the problems outlined, mechanical activity, Na- and K-content, and the binding of ouabain were determined at different frequencies in isolated left auricles of the guinea-pig. A ouabain concentration was chosen to provide a pure inotropic response at low frequencies. Based upon the results obtained, a hypothetical model will be discussed which involves the activity of the cardiac glycoside receptor (the Na-K-activated ATPase) in frequency-dependent actions of cardiac glycoside on heart muscle.

Some of these results were presented to the German Pharmacological Society (Bentfeld, Peters & Schreiner, 1976).

Methods

Mechanograms of left auricles isolated from guinea-pigs weighing about 300 g were recorded isometrically by means of a strain gauge; the auricles were preloaded with 0.5 g, and electrically stimulated by rectangular pulses of 4 ms duration, 3–6 V, and with frequencies as indicated below. The Tyrode solution (mM: NaCl 135, KCl 2.7, $CaCl_2$ 1.3, $MgCl_2$ 1.0, $NaHCO_3$ 12, NaH_2PO_4 0.21 and glucose 5.5) was aerated by a mixture of 95% O_2 and 5% CO_2; the temperature was kept at 32°C.

Sodium and potassium were determined by flame photometry. The tissue samples were divided in two parts, one of which was used for [^{3}H]-ouabain measurement (see below) the other for determination of the ions. After different incubation periods the auricles were removed, blotted by a standard procedure (Lüllmann & van Zwieten, 1967) ashed to dryness in 1 ml of a mixture of equal amounts of HNO_3 (65%) and $HClO_4$ (60%), and re-dissolved in 0.1N HCl (Zepf, 1966). For each set of experiments a calibration curve was established and the Na and K backgrounds were separately determined. Each muscle sample was subjected to a double determination. The size of the extracellular space (ECS) was taken from earlier experiments performed under identical conditions and amounted to 0.3 ml/g wet wt. in non-intoxicated and 0.26 ml/g wet wt. in contracted muscles (Lüllmann & van Zwieten, 1967). The cellular Na and K contents were calculated by subtracting the respective extracellular amounts from the total tissue contents, and are expressed as mmol/kg cell.

Ouabain binding was measured in the second part of each auricle by means of tritiated ouabain (sp. act. 10 Ci/mmol, NEN, Chicago). After blotting, the samples were dissolved in Soluene (Packard Instr.), and subjected to liquid scintillation counting. The concentration of [^{3}H]-ouabain determined in the tissue (corrected for by the amount present in the ECS) relative to medium concentration was expressed as tissue medium ratio (T/M ratio = concentration per g cell divided by concentration per ml of Tyrode solution).

Results

Effects on contractile force and binding of ouabain at different beat frequencies

The action of ouabain was investigated at stimulation frequencies, of 0, 1, 2, 4, and 5 Hz. The ouabain concentration of 0.4 µM proved to act purely inotropically at 1 Hz, but induced toxicity at the higher frequencies; the onset of both actions was accelerated with increasing frequencies. For any investigated beat frequency the mechanograms were recorded and the cellular Na and K content as well as ouabain binding were determined for time periods up to 270 minutes.

In the resting state the Na and K content of the auricles remained unchanged for the entire period of observation, as shown in Figure 1. The figure also depicts rate and amount of ouabain binding. After 270 min, the T/M ratio attained a value of about 1.4, but had not yet reached the final equilibrium level. Under resting conditions, the binding of ouabain proceeded comparatively slowly but nevertheless the glycoside was accumulated.

At 1 Hz, ouabain almost doubled the contractile force. The maximum inotropic response was reached within approximately 90 min (Figure 1). The ion content of the muscles remained unaltered by ouabain throughout the entire incubation period as compared

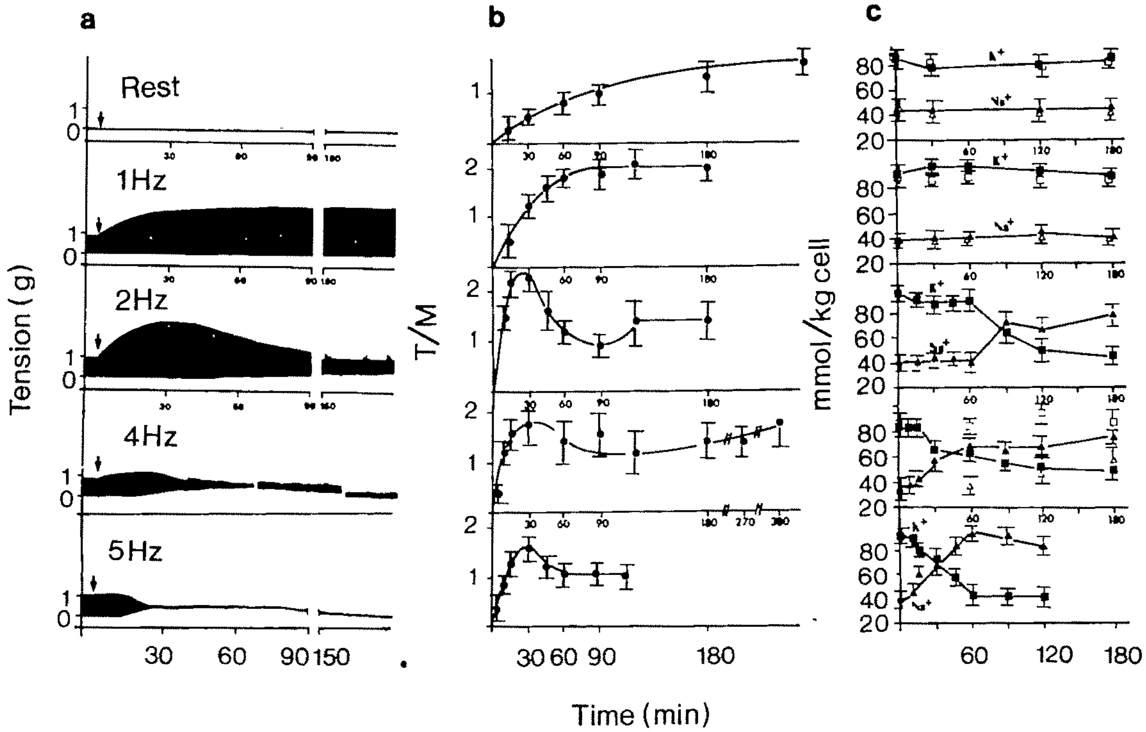

Figure 1 Influence of frequency of contraction on the effect of ouabain on contractile force, cellular Na and K concentration and binding of the drug to isolated left auricles of guinea-pig. (a) Ordinate scale: contractile tension (g); (b) ordinate scale: T/M-ratio (= radioactive ouabain per g tissue divided by radioactive ouabain per ml Tyrode solution); (c) ordinate scale: Na and K (mmol per kg cell). In (a), (b) and (c) abscissa scale: time (min). Open symbols = controls; solid symbols = in the presence of ouabain. Squares: cellular K; triangles: cellular Na. Reading from above, downwards the frequencies are: 1, 2, 4 and 5 Hz. Note that the abscissa scale is different for the recordings of mechanical activities. Points on the curves are mean values of at least 8 individual experiments. Vertical lines show s.e. means.

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to the untreated control muscles. A small but significant increase in the K content could be observed between 30 and 120 min when the heart muscle developed its maximum contractile force. As also demonstrated in Figure 1, the ouabain binding reached a plateau after about 120 min at a T/M ratio of 2.2. In comparison to the resting muscle the rate of binding was considerably faster and the amount of ouabain bound was higher.

At 2 Hz, ouabain at the same concentration produced a transient positive inotropic effect with a maximum increase of about 100% after 30 min of incubation. Thereafter the muscles displayed toxic signs, i.e. decay of maximum systolic tension, arrhythmia, and contracture (Figure 1). The cellular Na and K content remained constant for 60 min in spite of the continuous presence of ouabain. Then a progressive loss of K and a gain of Na could be observed which coincided with the occurrence of toxic signs (Figure 1). The ouabain binding proceeded at an even higher rate as compared to that at 1 Hz. The maximum binding (T/M of 2.3) was already attained within about 30 min of exposure to ouabain. But in contrast to the preceding experimental condition, the binding of ouabain at the higher frequency was not maintained but fell within 60 min to a T/M value of about 0.8, in spite of the continued presence of ouabain. Thereafter, the binding of ouabain slowly increased again (Figure 1). The reduction of the T/M values which indicates a release of previously bound ouabain, corresponded to the onset of toxicity.

At a beat frequency of 4 Hz, ouabain at a concentration of 0.4 μM, evoked only a small (about 15%) transient positive inotropic effect within 15 min (Figure 1). This response was followed by intoxication. During the short phase of increased contractile force, the Na and K content remained unaffected. Immediately upon the onset of intoxication, the auricles started to lose K and to gain Na. Initially, loss of K and gain of Na proceeded rapidly, but progressively slowed and final values for the cellular ion concentrations were approached between 180 and 270 min (Figure 1). Ouabain was bound at a rate similar to that observed at 2 Hz, the maximum T/M value was, however, reduced to 1.8, and subsequently declined to about 1.0 in the continued presence of ouabain. Thereafter, the T/M ratios slowly increased with time, reaching finally a value of 1.4 which is identical with the value obtained under resting conditions after the same time of exposure to ouabain.

At a beat frequency of 5 Hz, 0.4 μM ouabain no longer had a positive inotropic action, but immediately upon addition induced intoxication (Figure 1). Simultaneously the cellular Na content increased and K was lost. Both processes attained final equilibria for the Na and K distribution within 60 min of exposure of the auricles to the drug (Figure 1). The ouabain binding curve was quite similar to that obtained at 4 Hz but the peak value was found to be considerably lower. The subsequent decline of the T/M ratios reached its minimum also after about 60 min of incubation with ouabain (Figure 1).

Reversibility of binding and effects of ouabain

To test the reversibility of binding and the effects of ouabain, the auricles were incubated for 120 min with [^{3}H]-ouabain at a concentration of 4×10^{-7} M at three different frequencies. Thereafter the organs were transferred to a drug-free Tyrode solution. Contractile parameters, cellular Na and K content and T/M ratios (M is now concentration of [^{3}H]-ouabain in the Tyrode solution before the wash-out phase) were determined for time periods up to 180 minutes. The frequencies 0, 1 and 4 Hz were chosen since they provided three different situations as far as actions and binding of ouabain are concerned. As demonstrated in Figure 2, similarly shaped wash-out curves for ouabain were obtained in resting, strongly contracting and contractured auricles, although the amounts of ouabain bound before the beginning of the wash-out experiments were different under the three conditions studied (see uptake experiments and legend to Figure 2). Upon wash-out the tissue levels of ouabain declined to half their values in about 12 min, independent of the condition, and reached background values after about 100 minutes. The mechanograms in Figure 2 demonstrate that the inotropic response declined at a similar rate whereas the contractured muscle remained intoxicated although ouabain had been completely removed. In the latter case cellular loss of K and gain of Na proceeded irrespective of the release of ouabain from the tissue: at the beginning of the wash-out period the cellular contents of Na and K were 70 and 55 mmol/kg respectively. At the end of the 2 h wash-out period the respective values amounted to 100 mmol/kg for Na and 40 mmol/kg for K. In resting muscles and in muscles beating at 1 Hz no changes for the cellular ion concentrations could be observed during the uptake and wash-out periods.

Discussion

In order to find a link between the actions of cardiac glycosides on contractile parameters and on the transmembrane ionic transport system, the aim of the present investigation was to study the influence of frequency of contraction on glycoside binding to atrial tissue, and simultaneously the effects of cardiac glycosides on contractile force and cellular Na^+ and K^+ concentrations. Conditions were chosen which allowed one particular ouabain concentration, merely by an alteration of the stimulation frequency, to elicit (1) a sustained positive inotropic response, or (2)

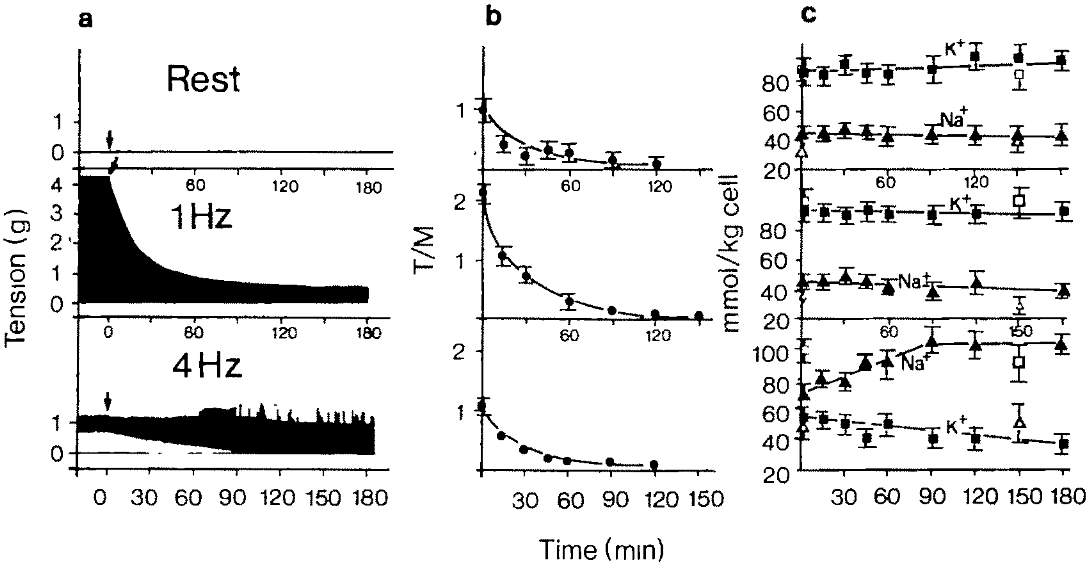

Figure 2 Reversibility of the effects of ouabain on contractile force and cellular Na and K concentrations and release of the drug from the tissue upon wash-out. (a) Ordinate scale: contractile tension (g); (b) ordinate scale: T/M-ratio; (c) ordinate scale: cellular Na and K concentrations. In (a), (b) and (c) abscissa scale: time (min). Arrows indicate beginning of the wash-out of ouabain. Points on the curves represent means of at least 6 individual experiments Vertical lines show s.e. means.

transitory positive inotropic effects followed by intoxication effects, or (3) exclusively toxic effects.

As can be seen in Figure 1, the mechanical responses obtained upon addition of ouabain were frequency-dependent. The time to maximum positive inotropic effect was about 60 min at a frequency of 1 Hz, 30 min at 2 Hz, and 15 min at 4 Hz, corresponding to about 3600 beats for these three conditions. This strong correlation between number of beats and time to positive inotropic maximum agrees well with the data of Wilbrandt *et al.* (1953), Sanyal & Saunders (1958), Gersmeyer & Holland (1963), Holland (1964) and Moran (1967). At 5 Hz the positive inotropic action is almost immediately overcome by toxic effects, and such an effect may also already be involved at 4 Hz. As far as the onset of intoxication is concerned, at 2, 4 and 5 Hz again, a beat-dependence becomes obvious for the negative inotropic phase as well as for the increase in diastolic tension: the former being visible after about 3600, the latter beginning after roughly 5000 beats. A similar beat-dependence for the onset of toxicity has been demonstrated by Weizsäcker (1913), Holland & Sekul (1961) and Gersmeyer & Holland (1963) (for review see Lee & Klaus, 1971).

For the rate of binding of ouabain a frequency-dependence can only be seen at 1 Hz in comparison to 2 Hz: under both conditions the binding maxima are attained after about 3600 beats. Possibly, this may still hold true for 4 Hz, since the T/M ratios at 15 and 30 min are not significantly different from each other (Figure 1). If the binding rates are compared to the influx of sodium per unit of time, the rate of binding at rest should also be included in the comparison. According to Shanes (1958) and Langer (1968) about 0.5 mmol Na/kg enter the cell per min under resting conditions. Each depolarization adds another 0.04 mmol/kg and increases the actual Na concentration by about 0.1%. Thus the following amounts of Na (which will have to be extruded from the cell by the Na-pump) enter the cells: rest 0.008; 1 Hz 0.04; 2 Hz 0.08; 4 Hz 0.16 and 5 Hz 0.20 mmol Na kg^{-1} s^{-1}. In resting muscles the equilibrium of binding of ouabain was attained after about 300 min which corresponds to a Na-flux of 150 mmol/kg cell. At 1, 2, 4 and 5 Hz, this value is attained within about 60, 30, 15 and 12 min, respectively. Except for the highest frequency this correlation agrees with the experimental results suggesting that the binding process might depend upon the Na-flux.

During the development of the positive inotropic effect no alterations of cellular Na- and K-concentrations were measurable. It seems much more obvious that as soon as cellular accumulation of sodium and loss of potassium occur the muscles develop an increase in diastolic tension.

Only at a beat frequency of 1 Hz does the development of the positive inotropic effect evoked by ouabain parallel the binding of the drug, and is associated with a slight and transient increase in the cellular K-concentration (which would rather reflect a stimulation than an inhibition of the ATPase). The cellular Na-concentration remains completely unaffected. The maximum positive inotropic effect is attained when the majority of binding sites is occupied by ouabain (T/M = 2). Data of Kuschinsky, Lüllmann & van Zwieten (1968), Godfraind & Lesne (1972), Erdmann & Hasse (1975) and Lüllmann *et al.* (1975a) suggest about 90% of the adsorbed ouabain to be specifically bound to a saturable compartment identical with the Na-K-ATPase.

An increase of the stimulation frequency to 2, 4 and 5 Hz progressively shortened the positive inotropic state, induced transitory maxima of binding of ouabain, and even reduced the maximum binding and positive inotropic effect (Figure 1). According to Brinkmann & Ravens (1976) the decline of contractile tension, which at higher beat frequencies follows the positive inotropism induced by cardiac glycosides, and the reduced positive inotropic effect, are correlated to a critical shortening of the action potential duration. The early repolarization phase seems to prevent a complete activation of contractile elements by calcium ions which become rebound to their binding sites (Lüllmann & Peters, 1976). Neither the binding maxima nor the following decay of ouabain binding correlates to changes in cellular Na- or K-concentrations (Figure 1). It is suggested that the release of ouabain, in spite of its continuous presence in the the bath may be caused by a lowered concentration of ATPase conformations which readily bind ouabain (e.g. depletion of ATP, accumulation of calcium).

The cellular loss of K and gain of Na at higher beat frequencies coincides with the increased diastolic tension. Arrhythmias may occur simultaneously. Decreased gradients for K and Na are accompanied by a lowered transmembrane potential, which in combination with a shortened action potential favours the occurrence of extrasystoles and arrhythmias. Similarly, during increase in diastolic tension an increase of cellular calcium concentration due to a decreased calcium outward transport was reported by Kasparek (1976). The cellular accumulation of calcium will in turn hinder relaxation and, according to Godfraind & De Pover (1976) will itself inhibit the Na-K-ATPase. Thus in spite of wash-out of ouabain, the Na-K-ATPase remains inhibited as can be seen in Figure 2. During the phase of contracture a slow binding of ouabain reappears, which may reflect a continuous accumulation of inorganic phosphate in the cytosol (Lee, Ju, Lee & Burstein, 1960). As is known from isolated ATPase preparations, in the presence of inorganic phosphate, Mg^{2+}, and Na, all ATPase molecules will occupy a binding conformation for cardiac glycosides and lose their transport activity (Schwartz, Lindenmayer & Allen, 1975).

Possible determinants for the rate of binding of ouabain

The specific binding site for cardiac glycosides is the Na-K-ATPase which upon binding cardiac glycosides, loses its ability to transport Na^+ and K^+ (Repke, 1964, for review see Schwartz *et al.*, 1975). During the pumping process the ATPase undergoes several conformational steps with different binding affinities for cardiac glycosides (Post, Kume, Tobin, Orcutt & Sen, 1969). The intracellular Na concentration seems to play a major role for the cycling frequency of the ATPase. The rate of binding of cardiac glycosides to the isolated enzyme increases with increasing Na concentrations (Schwartz *et al.*, 1975). For this reason it is tempting to correlate the amount of Na which enters atrial cells per unit of time to the activity of the ATPase and thus to the rate of binding of cardiac glycosides. Figure 1 shows that the rate of binding is enhanced at higher beat frequencies. The essential requirement, i.e. an increased intracellular Na concentration is, however, not fulfilled, indicating that according to the law of mass action the individual ATPase centre will not be stimulated by the increased frequency. Yet, the depolarization-dependent influx of Na will occur more frequently if the beat frequency is increased. Consequently, the ATPase is faced with the same concentration but a higher amount of Na^+ to be extruded. This necessitates additional cycling of the transport system, i.e. extra pumping activity. The question arises, whether in the intact cell increased frequencies provide conditions under which per unit time the concentration of ATPase-conformations with high affinity for cardiac glycosides is augmented.

An increase of the 'high affinity receptor' concentration would also explain the higher rate of formation of the ouabain-ATPase complex in contracting as compared to resting muscles. As outlined above, there is a close correlation between size of Na influx/min at rest and Na influx/min at different frequencies and the rates of binding of ouabain under these conditions and it may be suggested that also under *in vivo* condition the loading of the ATPase by Na^+ is responsible for the rate of onset of action and binding ouabain.

As a working hypothesis it is possible to offer a model accounting for the experimental observation that the actions of ouabain on atrial muscle under specific conditions are frequency-dependent. Since under control conditions the cellular Na concentration did not increase even at high beat frequencies (under the present conditions, up to 5 Hz), the ATPase seems to extrude the extra amount of Na in very short

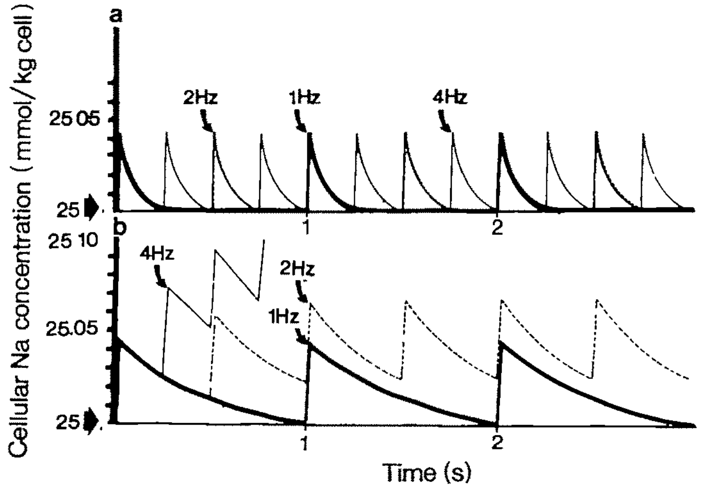

Figure 3 Hypothetical presentation of intracellular undulations of Na by means of cumulative Bateman functions (Dost, 1968) (a) Depicts control conditions; ordinate scale: cellular Na concentration in mmol/kg cell; abscissa scale: time (s). The upstrokes represent depolarization-dependent Na-influxes. The respective frequencies are indicated by arrows. (b) Presents the situation after partial inhibition of ATPase by ouabain. Depending on frequency, i.e. load of the inhibited ATPase, cellular Na concentration remains constant or increases. For details see text.

periods of time. The time necessary for the extra pumping activity of the ATPase is limited by the interval between two beats and thus should be less than 200 milliseconds. Above 5 Hz cellular Na starts to rise. This indicates that a critical beat frequency exists with respect to an overloading of the membrane ATPase. Hypothetically the undulations of the cellular Na concentrations at different frequencies are demonstrated in Figure 3.

The ATPase activity can be stimulated by increasing Na concentrations in the range between 1 and 100 mM (for review see Schwartz *et al.*, 1975), such that small alterations of the Na concentrations like those occurring during an action potential, do not significantly activate the pump. In conclusion, in intact cells, the ATPase activity appears to be governed rather by the frequency of electrical events than by altered cellular Na concentrations. A reduction of the number of available ATPase molecules should lead to a lower critical frequency at which the cellular Na concentration will rise, because the extrusion process demands more time to deal with the excitation Na influx in order to warrant an adequate end-diastolic Na concentration (Figure 3). Ouabain in fact reduces the critical beat frequency as shown above. Similar observations have been made by Issekutz (1915) and Gersmeyer & Holland (1963). The interpretation may be that, whenever the critical frequency is exceeded, due to reduction of the number of active ATPases, the cellular Na concentration progressively increases and the K concentration falls accordingly to an equilibrium value corresponding to the respective frequency (Figure 3).

Interaction of cardiac glycoside with Na-K-ATPase seems to induce conformational changes beyond the protein of the membrane bound enzyme (Lüllmann, Peters, Preuner & Rüther, 1975b). This change is thought to alter the characteristics of calcium binding to the plasmalemma resulting in an enhanced availability of coupling calcium which will improve the effectiveness of the excitation-contraction-coupling process and thus induce a positive inotropic effect (Lüllmann & Peters, 1976; 1977). In fact, experimental evidence exists that cardiac glycosides may alter the calcium binding characteristics of the Na-K-ATPase (Schwartz, 1976; Proppe, 1976). From the foregoing it may be suggested that the positive inotropic effect of ouabain does not result from an inhibited ion transport (cf. Figure 1). A possible mode of interaction between ouabain and the functional states of the ATPase is drawn schematically in Figure 4. The dual effects of ouabain on pump activity and on Ca binding sites are indicated.

While the rate of association of ouabain is determined primarily by the cycling frequency of the ATPase, the dissociation rates were found to be independent of the activity of the ATPase and of the situation of the muscle. As can be seen from the wash-out experiments in resting muscles and in muscles in a pure inotropic state, ouabain can dissociate from the ATPase, which continues the transport cycle and returns to its normal binding characteristics

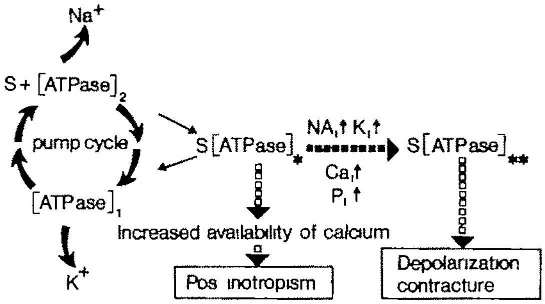

Figure 4 Hypothetical model depicting binding of ouabain to the Na-K-ATPase in intact cells and the interdependence between conformational alterations of the eynzyme and impairment of ion transport. S=ouabain; ATPase$_1$=enzyme in a conformational state with low affinity for cardiac glycosides; ATPase$_2$=enzyme with high affinity for cardiac glycosides; bent solid arrows indicate transition of additional conformational steps during one activity cycle; S [ATPase]$_*$=ouabain-ATPase complex; the conformation of the enzyme has changed, enzymatic and transport activities are reversibly blocked, availability of calcium is enhanced; Na$_i$; K$_i$, Ca$_i$= intracellular sodium, potassium, and calcium concentrations; Pi=inorganic phosphate concentrations; ↑=increase, ↓=decrease; S [ATPase]$_{**}$=further conformational change of the enzyme which eventually becomes irreversibly inhibited by lack of ATP or increased diastolic cellular Ca^{2+}-concentrations even if ouabain becomes removed from the ATPase. As long as the proportion of uninhibited ATPase molecules is sufficient to counteract Na influx at a given frequency, the increased availability of calcium underlying the positive inotropic action will be the only manifestation of the ouabain-ATPase interaction.

(Figure 4). The latter is supported by the finding that upon wash-out of ouabain the positive inotropic effect and the contractility concomitantly return to the control level (Figure 2), and the muscle again becomes susceptible to ouabain.

We gratefully appreciate the skilful technical assistance of Miss Susanne Quednau and the secretarial help of Mrs U. Pfeil.

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(Received September 27, 1976.
Revised February 4, 1977.)

Br. J. Pharmac. (1977), **61**, 29–37

SOME EFFECTS OF INTRAVENOUS PROSTAGLANDIN E_1 AND ENDOTOXIN IN YOUNG CHICKENS

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1 The effects of intravenous prostaglandin E_1 and endotoxin were studied in young chickens (11–17 days old).

2 At a thermoneutral ambient temperature (31°C), intravenous prostaglandin E_1 produced behavioural and electrocortical sleep, increased oxygen consumption and, after an initial fall, elevated body temperature. Below thermoneutrality (16°C), the initial hypothermic effect was more marked and oxygen consumption was lowered.

3 The soporific actions of prostaglandin E_1 were sufficient to counteract dexamphetamine-induced behavioural and electrocortical arousal and vocalization.

4 Intravenous injection of the O-somatic antigen of *Shigella dysenteriae* evoked, after a latent period, long lasting hyperthermia. This indicates that in young chicks the blood brain barrier is probably permeable to endotoxins.

Introduction

Horton (1964) demonstrated that intravenous prostaglandin E_1 produced behavioural sleep in young chicks. This action was presumed to be central since the blood-brain barrier is absent or not fully effective at this age (Waelsch, 1955; Bakay, 1956; Lajtha, 1957); indeed, tritiated prostaglandin E_1 penetrates into the brain after intravenous or intra-arterial injection in young chicks (Holmes & Horton, 1968). When infused into selected brain-sites to bypass the blood-brain barrier, prostaglandin E_1 induced behavioural sleep in young and adult chickens (Nisticò & Marley, 1973; Artunkal & Marley, 1974). The present paper describes more fully the effects of intravenous prostaglandin E_1 on behavioural, electrocortical and metabolic activities and on body temperature. Additionally, the effects of intravenous endotoxin on body temperature were investigated since failure of endotoxin, administered by this route, to elevate body temperature of adult hens (Pittman, Veale, Cockerham & Cooper, 1976) has been attributed to relative impermeability of the blood-brain barrier to endotoxins and/or the endogenous pyrogens produced by them. The effects of prostaglandins E_1, E_2 and of the O-somatic antigen of *Shigella dysenteriae* injected into the hypothalamus are compared in a subsequent paper with their effects when given intravenously.

Methods

Animals

Rhode Island Red pullets of 80–90 g were used (11–17 days old). They were housed under thermoneutral conditions i.e. at 33–34°C for the first week after hatching and for the following 2 weeks at 29–31°C.

Operative procedures

These were performed under halothane anaesthesia. Methods for implanting electrocortical recording electrodes, an intravenous jugular cannula and a thermistor placed subcutaneously between the scapulae have been described (Dewhurst & Marley, 1965; Allen & Marley, 1967). For injections into an internal carotid artery, retrograde injections were made down a polyethylene cannula inserted into the external carotid artery so that its tip lay at the junction with the common and internal carotid arteries.

Experimental procedures

Chicks were tested when recovery was complete, at least 24 h after the operative procedures. About 1 h before the control period, each chick was placed in a soundproof, environment-controlled experimental box with a one-way screen and facilities for external monitoring of body temperature and electrocortical activity. Electrocortical activity was automatically integrated at 1 min intervals, large amplitude potentials producing high integral counts and alert low voltage electrocortical patterns giving low integrals. Vocalization was recorded and quantitated by the same integrating method. Ambient temperature was maintained at 16±0.5°C or 30–31°C, i.e. below or within the thermoneutral range for chicks at this age (Freeman, 1963; Allen & Marley, 1967); relative humidity was maintained at approximately 60%.

Intravenous and intra-arterial injections were given via a length of polyethylene tubing which passed through the roof of the box to connect with the implanted cannula. Body temperature was monitored continuously on a Grant miniature recorder; results are expressed as actual body temperatures rather than changes in body temperature. Oxygen consumption was measured as described by Allen & Marley (1967).

Drugs

Those used were prostaglandin E_1 (Upjohn & Co. Ltd.), O-somatic antigen of *Shigella dysenteriae* (W.H.O.) and dexamphetamine sulphate (S.K. & F. Ltd.). Prostaglandin E_1 was dissolved in ethanol and sodium carbonate (1 mg prostaglandin E_1 in 0.1 ml 95% ethanol and 0.9 ml sodium bicarbonate (0.2 mg/ml)) and prepared freshly for each experiment.

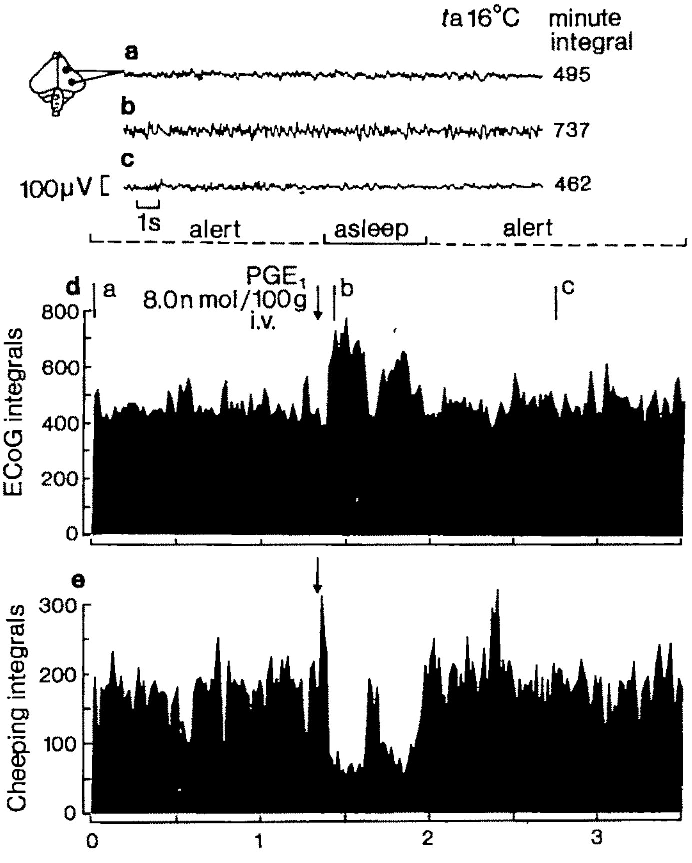

Figure 1 Effects of prostaglandin E_1 (PGE_1) on electrocortical activity and cheeping. Records of electrocortical activity (a–c; upper part) and of integrated electrocortical activity (d; middle) and cheeping (e; lower part). (a–c) Electrocortical activity recorded at times indicated in (d). (a), Control alert electrocortical activity; (b), activity of larger amplitude and slower frequency induced by prostaglandin E_1: (80 nmol/100 g i.v.) coincident with behavioural sleep and a reduction in cheeping (expressed as integrals, e). (c and d), Return of electrocortical activity and integrals to their pre-injection values, respectively. *ta*, ambient temperature, 16°C

Results

Behaviour, cheeping and electrocortical (ECoG) activity

Horton's (1964) finding that intravenous prostaglandin E$_1$ induced behavioural sleep was confirmed in 6 chicks at a thermoneutral ambient temperature (31°C) and in 14 chicks tested below thermoneutrality (ambient temperature 16°C). The chicks either squatted or stood in a 'tripod' position with the head lowered, the beak resting on the ground, the eyes closed and the wing and tail feathers relaxed. Additionally prostaglandin E$_1$ induced the electrocortical changes of slow wave sleep i.e. large amplitude (>100 µV) slow frequency (2 to 4 Hz) waves. As evident in Figure 1, taken from a chick maintained at an ambient temperature of 16°C and following prostaglandin E$_1$ (8 nmol/100 g i.v.), the control alert ECoG (Figure 1a) changed to the large amplitude, slower frequency pattern associated with sleep (Figure 1b), integrals of ECoG activity being doubled during this period (Figure 1d). Behavioural and electrocortical sleep lasted approximately 30 min, and there was a return to alert ECoG activity on recovery (Figure 1c) with reduction in ECoG integrals. As shown in Figure 1e, loud 'distress' cheeping, which occurs on exposure to cold i.e. 16°C (Collias & Joos, 1953), was substantially diminished during the behavioural and electrocortical sleep

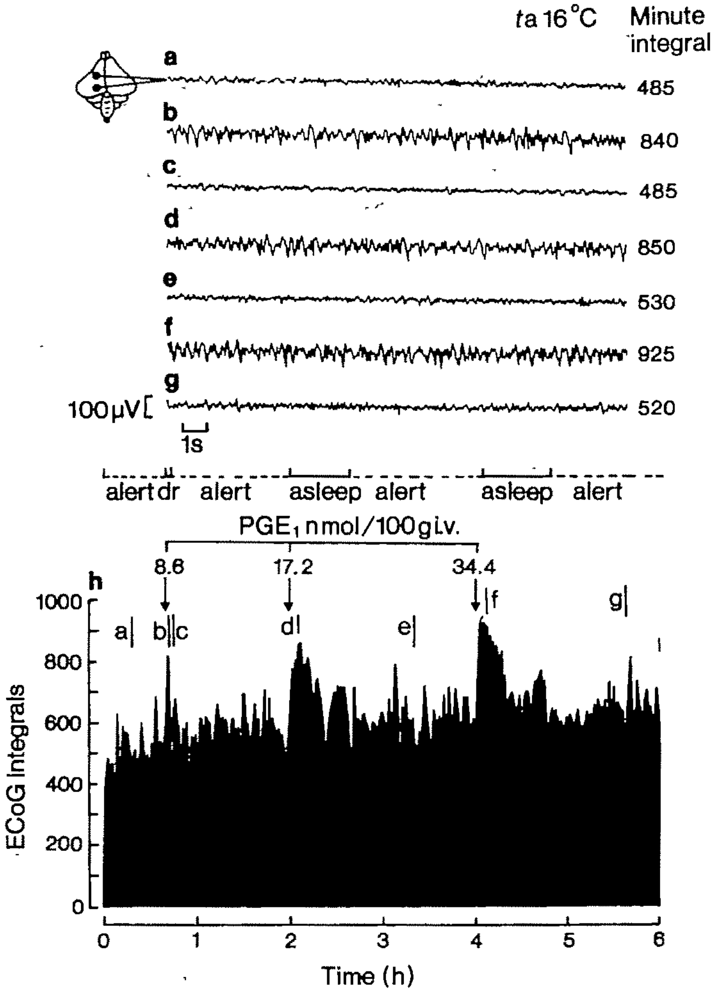

Figure 2 Effects of prostaglandin E$_1$ (PGE$_1$) on electrocortical activity (a–g), recorded at times indicated in lower part of figure. Increasing doses of prostaglandin E$_1$ (8.6, 17.2 and 34.4 nmol/100 g i.v.) produced similar changes in amplitude and frequency of electrocortical activity (b, d and f) but the changes were of increasing duration (h). ta, ambient temperature 16°C; dr, drug administered at time indicated.

induced by prostaglandin E_1. In Figure 2, taken from an experiment with another chick at an ambient temperature of 16°C, the electrocortical slow wave sleep changes (Figure 2b,d,f) induced by three doses of prostaglandin E_1 (8.6, 17.2, 34.4 nmol/100 g i.v. respectively) are illustrated, together with the return to alert electrocortical activity (Figure 2c,e,g) on recovery from each of the doses. While the maximum increase in ECoG integrals was similar for the three doses (Figure 2h), the durations during which these were elevated were dose-dependent (Figure 2h). However, mean electrocortical changes (Figure 3) indicated that it was the increase in integrals that was dose-dependent rather than the duration of effect.

The soporific actions of prostaglandin E_1 were intense, a point generally overlooked in comparison to its pyrexic actions, sufficiently so to counteract the central excitant effects of dexamphetamine and *vice versa*. Thus, Figure 4a–e illustrates that the electrocortical and behavioural sleep following prostaglandin E_1 (8.6 nmol/100 g i.v.), were converted to electrocortical (Figure 4c and e) and behavioural arousal by dexamphetamine (2 μmol/100 g i.v.), which in turn were replaced by sleep induced by prostaglandin E_1 (8.6 and then 17.2 nmol/100 g i.v.; Figure 4d and e). Amphetamine also elicits vocalization ('twittering') quantitatively different from that evoked by cold. As shown in Figure 5 taken from an experiment in which the chick exhibited little vocalization during the control period, cheeping was substantially increased by dexamphetamine (2 μmol/100 g i.v.) integrals of cheeping increasing from about 80–100/min to 250–330/minute. Cheeping was sustained for 20 min but was then attenuated by prostaglandin E_1 (16 nmol/100 g i.v.) only to return as the soporific effects of prostaglandin E_1 abated.

Body temperature

At a thermoneutral ambient temperature (31°C), prostaglandin E_1 (16, 32, 128 or 256 nmol/100 g) given either intravenously (9 chicks) or intra-arterially directly to the head (2 chicks) at first produced a dose-dependent hypothermia of between 1° and 2.5°C followed by an elevation of body temperature (Figure 6a and b). In 21 chicks below thermoneutrality (16°C) the initial hypothermic effect was more marked (compare Figure 6c and e).

Oxygen consumption

In the 9 chicks tested at thermoneutrality (31°C), mean oxygen consumption was elevated by 23 to 34% (see Table 1, Figure 6d) by prostaglandin E_1 (16, 32,

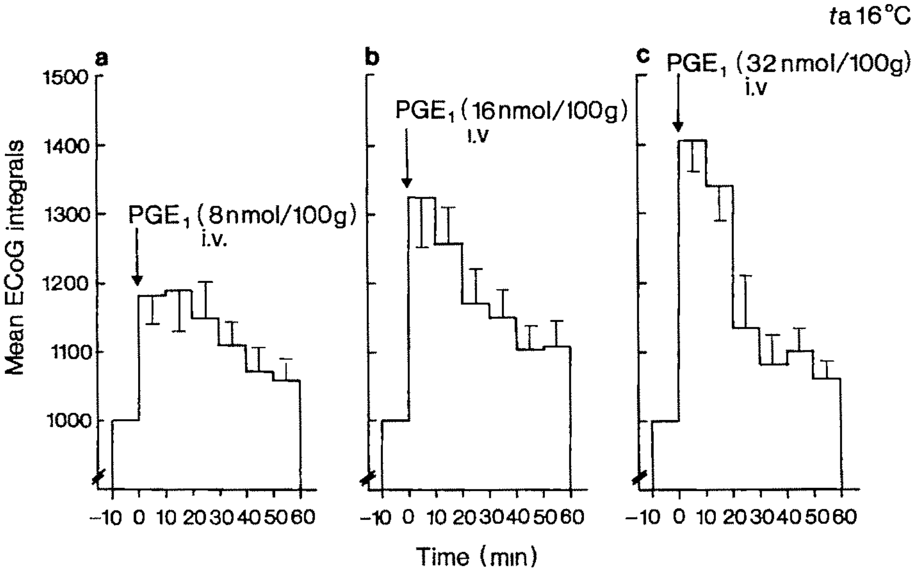

Figure 3 Mean effects of prostaglandin E_1 (PGE_1, 8, 16 and 32 nmol/100 g i.v.) on 10 min integrals of electrocortical activity. Vertical bars indicate s.e. mean. Mean control electrocortical integrals for each chick were normalized to 1000. (a) $n=14$, (b) $n=12$, (c) $n=3$, *ta*, ambient temperature, 16°C.

Table 1 Mean percentage changes, with s.e. mean in oxygen consumption due to intravenous prostaglandin E$_1$ (PGE$_1$) in chicks at an ambient temperature (*ta*) of either 31° or 16°C

Dose PGE$_1$ (nmol/100 g i.v.)	n	*ta* *31°C*	n	*16°C*
4			5	−24.24±5.1
8			9	−26.12±3.3
16	4	+23.13±1.4	6	−36.14±2.3
32	3	+23.95±4.8		
64	2	+34.5 ±9.5		

(+), increases and (−), decreases in oxygen consumption.

128 or 256 nmol/100 g i.v.). In contrast, for the 20 chicks tested below thermoneutrality (16°C), prostaglandin E$_1$ (8 or 16 nmol/100 g i.v.) produced an immediate marked fall in mean oxygen consumption of from 24 to 36% followed by a slight overshoot above control values (Table 1, Figure 6f); recovery with overshoot of oxygen consumption preceded recovery of body temperature.

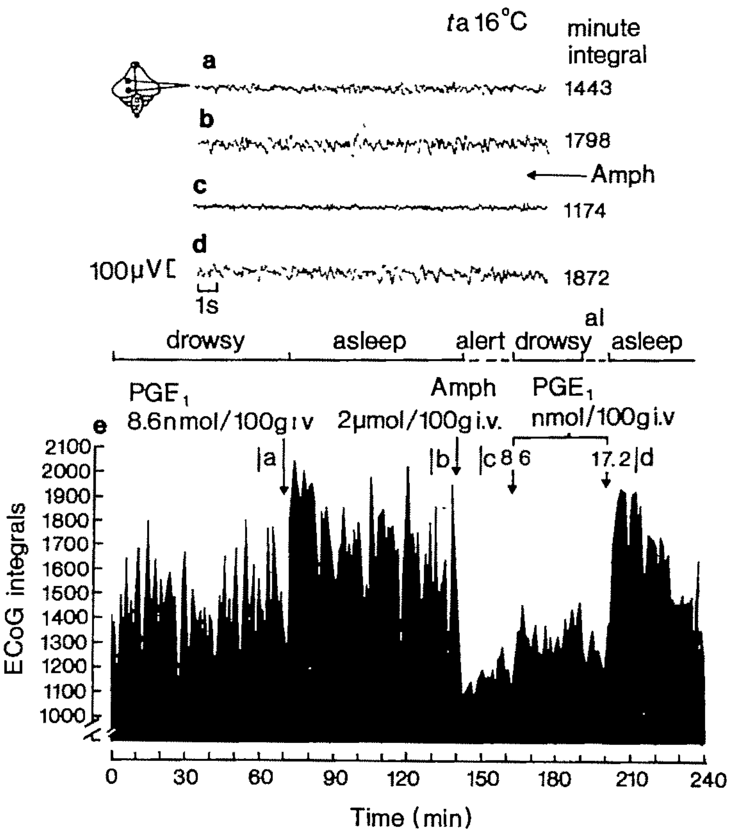

Figure 4 Reversal of the depressant effects of prostaglandin E$_1$ (PGE$_1$) (8.6 nmol/100 g i.v.) on electrocortical activity by dexamphetamine (Amph, 2 µmol/100 g i.v.) and of the excitant effects of dexamphetamine by a total of 25.8 nmol/100 g i.v. prostaglandin E$_1$. al, alert; *ta*, ambient temperature 16°C.

Effects of O-somatic antigen of Shigella dysenteriae *on body temperature*

The O-somatic antigen of *Shigella dysenteriae* (6 µg/100 g i.v.) evoked long-lasting hyperthermia commencing after a latency of approximately 1 h; ambient temperature was 31°C. Maximum elevation of mean body temperature (4 chicks) of 1.15°C was reached 4.5 h after injections and 4.5 h later it was still 0.6°C above the pre-injection values (Figure 7); in contrast, in saline- or vehicle-injected controls there was no elevation of body temperature. There was no evidence of an initial hypothermic response.

Discussion

The behavioural sleep evoked in chicks with intravenous prostaglandin E_1 (Horton, 1964) was shown in the present experiments to be accompanied by appropriate electrocortical changes. These phenomena, dose-dependent inasmuch as larger doses produced correspondingly longer effects, were assumed to be central in origin since similar effects ensued after intrahypothalamic injection (Artunkal, Marley & Stephenson, 1977). These soporific effects were sufficiently intense to replace amphetamine-evoked arousal with behavioural and electrocortical sleep; additionally, cheeping elicited by cold or by amphetamine i.e. by a physiological or a chemical stimulus was suppressed during sleep induced by prostaglandin E_1.

The effects on oxygen consumption and body temperature varied according to ambient temperature. At thermoneutrality (31°C), intravenous prostaglandin E_1 elevated oxygen consumption and after an initial fall, elevated body temperature; in contrast, below thermoneutrality it lowered both. Excluding the initial fall in body temperature at thermoneutrality these effects were similar to those of prostaglandin E_1 injected into the hypothalamus of young chicks and together with the behavioural effects, were assumed therefore to be central in origin. In 2–3 day old chicks, approximately 1/50th of an intravenous injection of

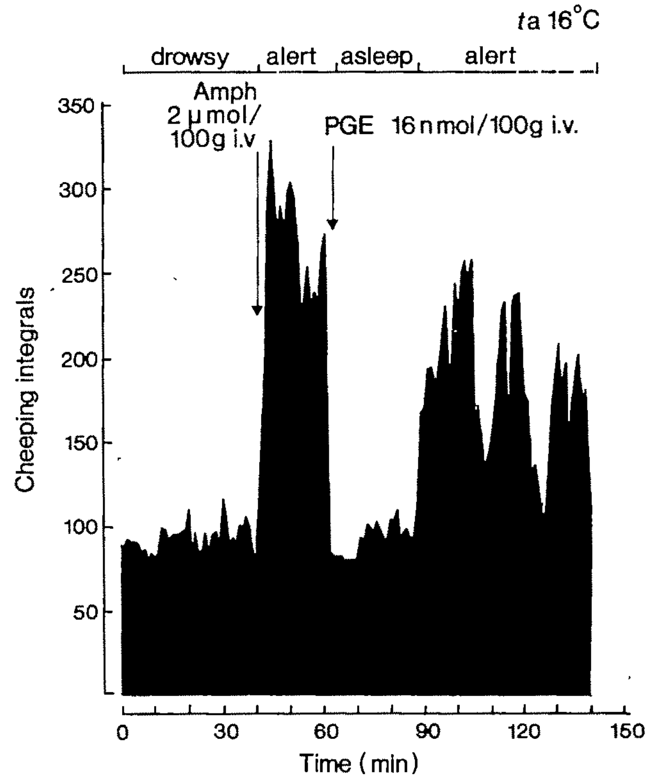

Figure 5 Suppression of dexamphetamine (Amph, 2 µmol/100 g i.v.)-induced vocalization by prostaglandin E_1 (PGE_1, 16 nmol/100 g i.v.). The effects of prostaglandin E_1 persisted for about 30 minutes. *ta*, ambient temperature 16°C.

PGE$_1$ (2 μg) was recovered in the brain 1 min after injection (Holmes & Horton, 1968). Assuming similar penetration for 11–17 day old chicks, then in the present study 0.1 to 0.5 nmol would be expected to enter the brain within 1 min of injection. This compares with our usual intrahypothalamic dose of 14.3 nmol (Artunkal & Marley, 1974; Artunkal *et al.*, 1977). Nevertheless, while the effects of intravenous prostaglandin E$_1$ on oxygen consumption (and body temperature) resembled those seen after intrahypothalamic injection a peripheral site of action cannot be entirely excluded since it has been reported that in 9 week old chicks, prostaglandin E$_1$ (8.5 nmol/100 g i.v.) produced total inhibition of

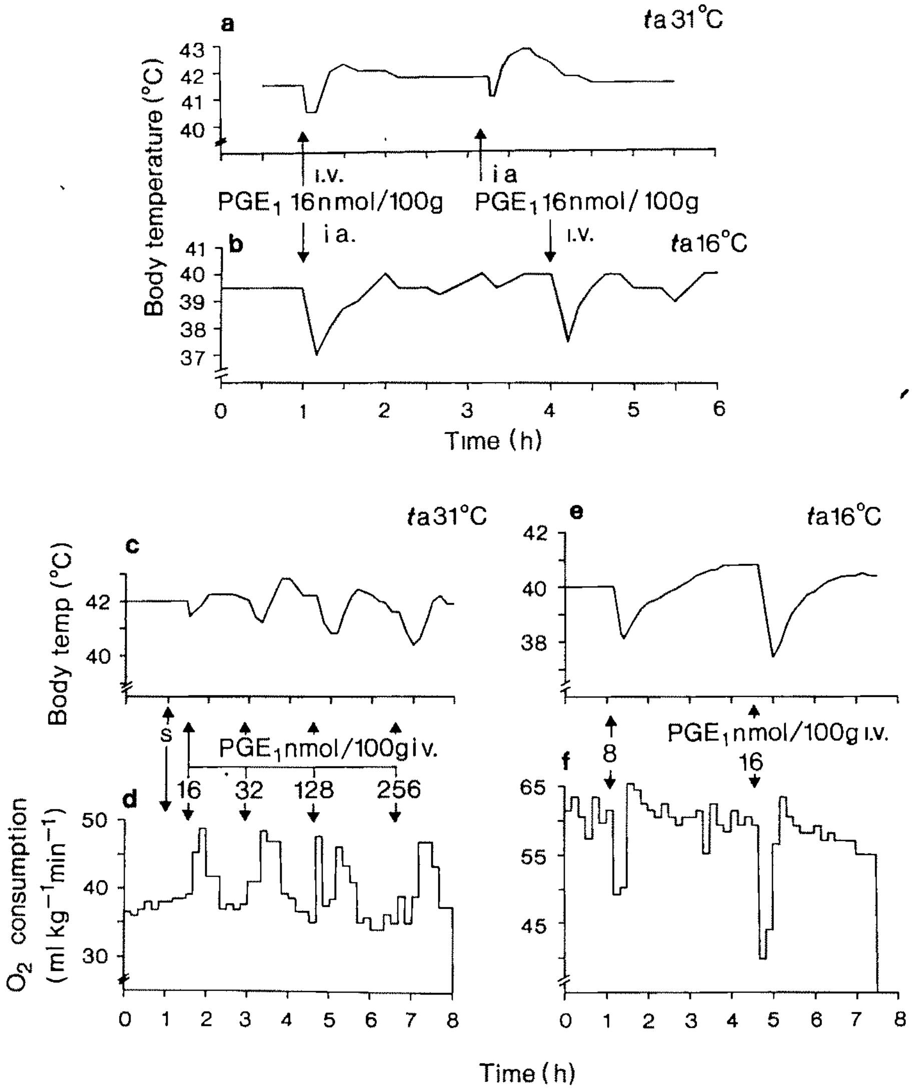

Figure 6 Representative traces comparing the effects of intra-arterial and intravenous prostaglandin E$_1$ (PGE$_1$) on body temperature of a chick at an ambient temperature (*ta*) of 31°C (a) and 16°C (b). Reducing ambient temperature to 16°C potentiated the initial hypothermic effect. At thermoneutrality (31°C), lowering of body temperature (c) was associated with an elevation of oxygen consumption (d), whereas below thermoneutrality (16°C), the initial hypothermic effect of prostaglandin E$_1$ (c) was accompanied by a fall in oxygen consumption (f).

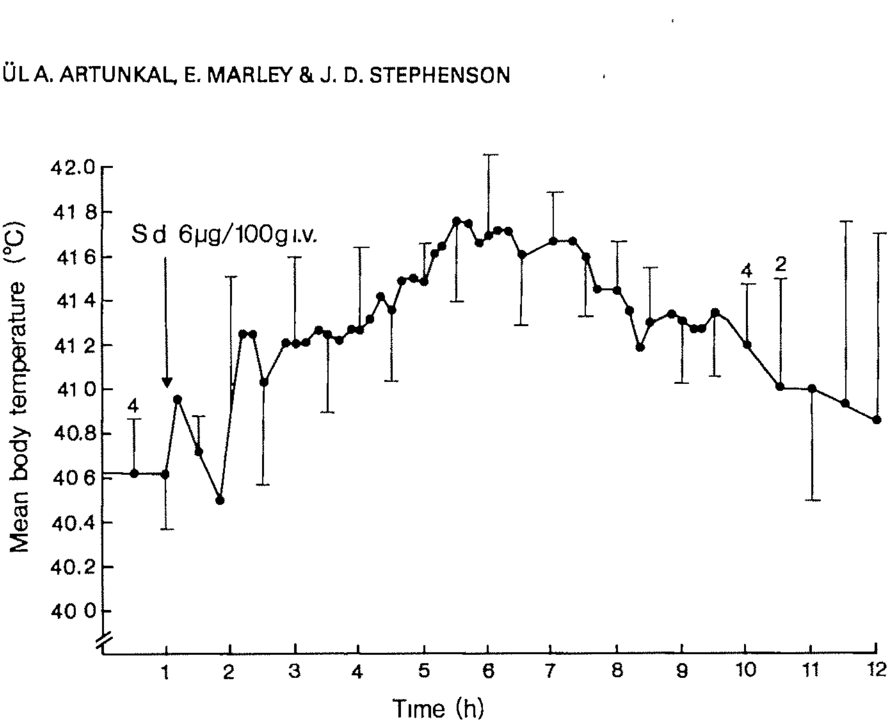

Figure 7 Mean effect of the O-somatic antigen of *Shigella dysenteriae* (S d. 6 µg/100 g i v) on body temperature of 4 chicks (last 4 mean values are from 2 chicks only). Vertical bars represent s.e. mean.

lipolysis induced by placing chicks in an ambient temperature of 2–3°C for 4 h (Wagner, Peterson & Cenedella, 1971). The relevance of this is difficult to assess since prostaglandin E_1 did not affect basal lipolysis at thermoneutrality (Wagner *et al.*, 1971) and under the present experimental conditions (2–3 week old chicks at 16°C), plasma non-esterified fatty acid concentration was not elevated (Marley & Stephenson, 1975).

Prostaglandins of the E series are thought to be the mediators of endotoxin fever (for references, see Feldberg, 1975). Since intravenous injections of endotoxins evoke hypothermia in rats (Feldberg & Saxena, 1975) and adult chickens (Pittman *et al.*, 1976), in contrast to the hyperthermia noted after their central administration, it has been suggested that the blood brain barrier of these species is relatively impermeable to endotoxins. Indeed, no convincing evidence of either endogenous or exogenous pyrogens entering the brain from the periphery has been obtained (Milton, 1976). The finding that in young chicks, in which the blood brain barrier is immature, intravenous endotoxin evoked hyperthermia suggests that at this age in this species endotoxin did enter the brain. Assuming that prostaglandin E_1 mediates endotoxin fever and that prostaglandins penetrate the brain of young chicks then the production of hypothermia after its intravenous injection below thermoneutrality seems paradoxical; this aspect is studied in the ensuing paper.

The authors thank W.H.O. and Dr J.E. Pike (Upjohn & Co. Ltd.) for gifts of O-somatic antigen of *Shigella dysenteriae* and prostaglandin E_1 respectively. We are also grateful to the Bethlem Royal and Maudsley Hospitals Research Fund for financial support.

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(Received October 19, 1976.
Revised April 7, 1977.)

Br. J. Pharmac. (1977), **61**, 39–46

SOME EFFECTS OF PROSTAGLANDINS E_1 AND E_2 AND OF ENDOTOXIN INJECTED INTO THE HYPOTHALAMUS OF YOUNG CHICKS: DISSOCIATION BETWEEN ENDOTOXIN FEVER AND THE EFFECTS OF PROSTAGLANDINS

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1 Prostaglandins E_1 and E_2 elevated body temperature of young chicks when injected into the hypothalamus at thermoneutrality (31°C). In contrast, they lowered body temperature when so injected below thermoneutrality (16°C): the relation of the fall in body temperature to increased heat loss and decreased heat production was examined.

2 The above effects below thermoneutrality were potentiated by pretreatment with inhibitors of prostaglandin synthetase and possible reasons for this potentiation are given.

3 The 'O'-somatic antigen of *Shigella dysenteriae* consistently evoked hyperthermia when injected into the hypothalamus, irrespective of whether the chicks were within or below thermoneutrality.

4 Pretreatment with prostaglandin synthetase inhibitors failed to prevent the onset of endotoxin fever; however, duration of the fever, induced by intrahypothalamic injection of the O-somatic antigen of *Shigella dysenteriae* was reduced.

5 The intrahypothalamic injection, below thermoneutrality of prostaglandins E_1, E_2, noradrenaline, 5-hydroxytryptamine or carbachol reversed endotoxin fever, inducing even substantial falls in body temperature.

6 While the results cast some doubts on the role of prostaglandins of the E series as mediators of endotoxin fever in chicks, they cannot be eliminated as mediators until the significance of the reduction in duration of the pyrexic response by indomethacin and 5,8,11,14-eicosatetraynoic acid, and the degree of synthesis inhibition attained, are known.

Introduction

Prostaglandins are present in mammalian and avian tissues, their concentrations in those of chickens, including the brain, mostly exceeding those in mammals (Horton & Main, 1967; Karim, Hillier & Devlin, 1968). Prostaglandins of the E series are generally thought to be mediators of endotoxin fever because, for example, they raise body temperature when injected intraventricularly, they appear in cerebrospinal fluid during endotoxin and lipid A fever in mammals, but disappear from it when fever is controlled by antipyretic drugs which inhibit prostaglandin synthesis (for references see Feldberg, 1975). However, there are significant exceptions to this relation. For example, hypothermic effects of intraventricular prostaglandin E_1 and E_2 injection have been demonstrated over a wide range of ambient temperatures in Echidna, *Tachyglossus aculeatus* (Baird, Hales & Lang, 1974). Prostaglandin E_1 injected into the hypothalamus of young chicks below thermoneutrality also lowered body temperature (Artunkal & Marley, 1974) and indeed, suppressed fever produced by the 'O'-somatic antigen of *Shigella dysenteriae* (Artunkal, Marley & Stephenson, 1975).

The present paper extends these preliminary findings and includes an investigation into the mechanism whereby prostaglandins lower body temperature in young chicks and the interactions between the 'O'-somatic antigen of *Shigella dysenteriae*, E prostaglandins, inhibitors of prostaglandin synthesis and putative transmitters within the hypothalamus.

Method

Animals

Rhode Island Red pullets of 80–90 g were used

(11–17 days old). They were housed under thermoneutral conditions i.e. at 33–34°C for the first week after hatching and for the following 2 weeks at 29–31°C.

Operative procedures

All operative procedures were performed under halothane anaesthesia. Methods for implanting an intrahypothalamic cannula, an intravenous jugular cannula, a carotid arterial cannula and a thermistor placed subcutaneously between the scapulae have been described (Dewhurst & Marley, 1965; Allen & Marley, 1967; Marley & Stephenson, 1970): leg temperature was recorded from a thermistor taped to the tarsometatarsal region of a hind limb.

Experimental procedure

Chicks were tested when recovery was complete, at least 24 h after the operative procedures; only those chicks which had fed since the operation, as indicated by the presence of food in the crop, were tested. About 1 h before the control period, each chick was placed in a soundproof, environment-controlled experimental box with a one-way screen and facilities for external monitoring of physiological temperatures. Ambient temperature was maintained at 16±0.5°C or 30–31°C i.e. below or within the thermoneutral range for chicks of this age (Freeman, 1963; Allen & Marley, 1967); relative humidity was maintained at approximately 60%.

Intracerebral injections were given via a length of polyethylene tubing which passed through the roof of the box and was connected externally to a 10 µl Hamilton syringe; drug injections (1 µl) were given over a period of 1 minute. Cannulae positions were subsequently located histologically. Body and leg temperatures were monitored continuously on Grant miniature temperature recorders. In experiments in which leg temperature was recorded, the chick was supported in a rubber cradle through which the legs protruded so that it was not able to squat to prevent heat loss.

For recording carbon dioxide elimination the chick was placed in a 1 litre chamber, maintained at 16°C through which CO_2-free air, also at 16°C, was passed at a rate of 1 l/minute. Percentage CO_2 in the expired air, measured with an infra-red analyser (Hartmann & Braun) was recorded continuously. Oxygen consumption was measured as described by Allen & Marley (1967). Mean arterial blood pressure was monitored on a Devices polygraph.

Drugs

These were: prostaglandins E_1 and E_2 (Upjohn Co. Ltd.), indomethacin (Merck, Sharp & Dohme, Ltd.), 'O'-somatic antigen of *Shigella dysenteriae* (W.H.O.), 5,8,11,14-eicosatetraynoic acid (ETA, Roche Ltd.), carbamylcholine chloride, 5-hydroxytryptamine maleate, (−)-noradrenaline. (−)-Noradrenaline base was dissolved in equimolar HCl immediately before use, prostaglandins E_1 and E_2 were dissolved in ethanol and sodium carbonate (1 mg prostaglandin E_1 in 0.1 ml 95% ethanol and 0.9 ml of a 0.02% w/v Na_2CO_3 solution) and prepared freshly for each experiment, indomethacin was dissolved in 0.8% w/v $NaHCO_3$ solution. Other drugs were dissolved in 0.9% w/v NaCl solution (saline).

Results

Effects of prostaglandins E_1 and E_2

At a thermoneutral ambient temperature (31°C), prostaglandin E_1(14.3 nmol) injected into the hypothalamus of young chicks elevated body temperature (Figure 1a), the increase in body temperature commencing approximately 10 min after injection with a mean maximum increase (1.1°C) occurring after 140 minutes. Injection of saline or prostaglandin vehicle (each 1 µl) did not change body temperature by more than ±0.1°C (6 chicks). Four hours after prostaglandin E_1 injection, the mean elevation was 0.7°C, the duration of the pyrexic response ranging in individual chickens from 5 to 6 hours. After injection, chicks slept either erect or squatting with the wings applied closely to the trunk. Respiratory rate was elevated to about 60/min but not to the extent that gular flutter occurred. Prostaglandin E_2 (14.3 nmol) injected into the hypothalamus elevated body temperature a mean maximum of 1.05°C (3 chickens).

Below thermoneutrality (16°C), injections of prostaglandin E_1 (14.3 nmol) into the hypothalamus lowered body temperature (Figure 1b). The fall in body temperature commenced immediately after the injection with a mean maximum decline in 6 chicks of 2.45°C±0.9°C being obtained between 70 and 120 min (mean 90 min); recovery occurred within 110–170 min (mean 140 min) of infusion. Sleep was much less marked than in tests at thermoneutrality and chicks squatted (hindering heat loss) rather than stood. The hypothermic effect of an equimolar dose of prostaglandin E_2 (14.3 nmol) was greater than that of prostaglandin E_1, body temperature falling a mean maximum of 3.85°C (2 chicks) at 60 minutes. In one chick, body temperature recovered within 3 h of the injection, whereas in the other, recovery occurred after 5 h but was then followed by an 'overshoot' of 1.5°C lasting a further 5 h before body temperature returned to normal.

The cause of this unexpected hypothermia was investigated by determining the actions of prosta-

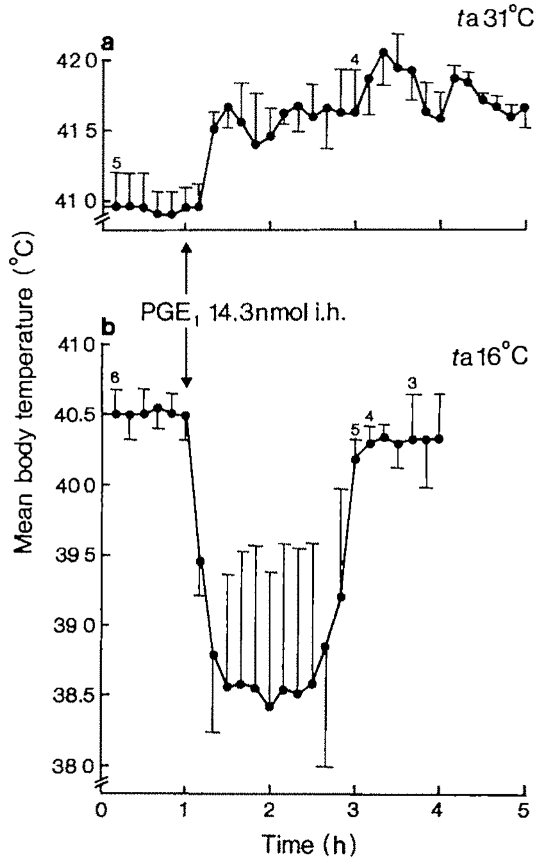

Figure 1 Mean effects of intrahypothalamic injection (i.h.) of prostaglandin E$_1$ (PGE$_1$, 14.3 nmol) at ambient temperatures (t_a) of (a) 31° and (b) 16°C. In this and Figures 3 and 4, points connected with continuous line indicate means; vertical bars indicate s.e. mean and the number of chicks used is shown by the numerals; where no numeral is shown, the last number appertains.

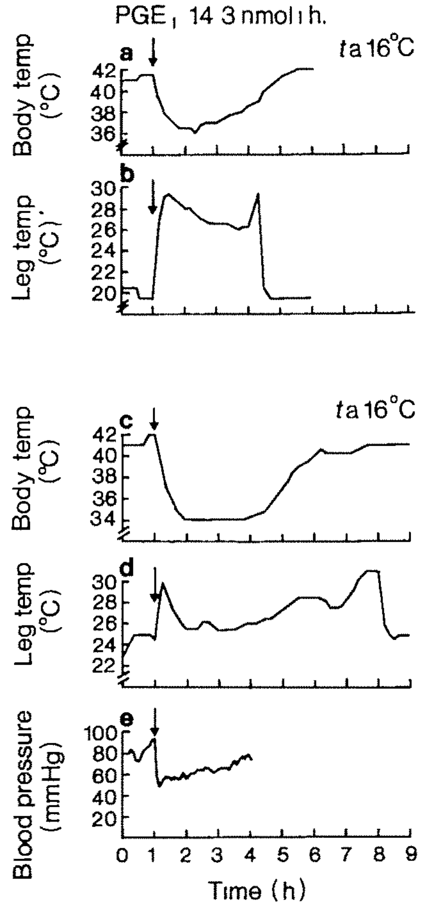

Figure 2 Effects of intrahypothalamic injection (i.h.) of prostaglandin (PGE$_1$, 14.3 nmol) on body temperature, leg temperature and mean arterial blood pressure at an ambient temperature (ta) of 16°C. In both chicks, the fall in body temperature (a,c) was associated with a related increase in leg temperature (b,d) more conspicuous in (b) and indicative of vasodilatation. The increased leg temperature (d) after prostaglandin E$_1$ was associated with a decline in arterial blood pressure (e).

glandin E$_1$ on heat loss and heat production mechanisms.

Heat loss. An increase in heat loss was inferred from the increase in temperature of the unfeathered lower limbs (hereafter, leg temperature) following injections of prostaglandin E$_1$ into the hypothalamus. It should be noted that covering the exposed lower limbs by squatting, reduces heat loss of adult fowls by one-third, (Deighton & Hutchinson, 1940). Since squatting alone elevated leg temperature by 3.0.°C, the experiments were performed with the chick suspended from a cradle (see Methods). Prostaglandin E$_1$ (14.3 nmol) injected into the hypothalamus caused a mean maximum increase in leg temperature of 6.5°C together with a mean maximum fall in body temperature of 5.5°C (4 chicks). The time-courses of the reciprocal responses were roughly similar although the rise in leg temperature was invariably more rapid than the fall in body temperature (Figure 2); the recovery of body temperature was preceded by that of leg temperature (Figures 2a,b) or *vice versa* (Figure 2c). The greater fall in mean body temperature that occurred in these experiments than is shown by chicks that were allowed to squat (5.5°C compared with 2.1°C) is presumably due to the inability of these chicks to reduce heat loss from the lower limbs.

The elevation in leg temperature was assumed to be secondary to general vasodilatation and was reflected in a reduced blood pressure. Thus prostaglandin E_1 (14.3 nmol) injected into the hypothalamus lowered mean blood pressure from 93 to 48 mmHg, while at the same time, leg temperature increased from 25° to 30°C (Figure 2e); body temperature began to fall during this period. Blood pressure gradually recovered over the ensuing 3 h whereas restoration of body temperature and leg temperature took considerably longer. These results, confirmed in 3 other chicks, support the suggestion that prostaglandin E_1 injected into the hypothalamus produced vasodilatation.

Heat production. Prostaglandin E_1 (14.3 nmol) injected into the hypothalamus of 8 chicks lowered CO_2 elimination within 5 min of injection from 56.43 ± 3.38 ml kg^{-1} min^{-1} to 33 ± 2.49 ml kg^{-1} min^{-1} ($P < 0.005$); the mean maximum decrease in temperature was 3.2°C. Oxygen consumption, measured at 100% humidity, was reduced in 6 chicks from 56.4 ml kg^{-1} min^{-1} to means of between 46.5 and 47.0 ml kg^{-1} min^{-1} over the ensuing 40 min; mean body temperature fell 2.2°C.

Effects of prostaglandin E_1 after indomethacin

Within thermoneutrality (31°C), indomethacin (1.4 µmol/100 g i.v. 30 min previously) delayed onset of the mean rise in body temperature to intrahypothalamic injection of prostaglandin E_1 (14.3 nmol) by approximately 70 min (compare Figure 3 with Figure 1a); the mean increase in body temperature of 1.05°C (4 chicks) was not significantly different from that elicited by prostaglandin E_1 alone. Despite considerable variations in the effects of prostaglandin E_1 after indomethacin, it appeared to attenuate the duration of the pyrexic response. Thus, following indomethacin, the mean duration of the pyrexic response was 4 h, at which time, body temperature after prostaglandin E_1 alone was still 0.7°C above the control values.

In contrast, the effects of indomethacin (1.4 µmol/100 g i.v. 30 min previously) below thermoneutrality (16°C) were clear-cut, the hypothermic effects of prostaglandin E_1 (14.3 nmol) being intensified and prolonged (Figure 3b). Thus the mean maximum fall in body temperature after intrahypothalamic injection of prostaglandin E_1 in 6 chicks was 5.4°C, significantly greater than after prostaglandin E_1 alone ($P < 0.025$); body temperature returned to pre-injection values approximately 4 h (225 ± 30 min, mean ± s.e. mean) after the injection compared to just under 2 h (108 ± 20 min) after prostaglandin E_1 alone ($P < 0.01$). Similar effects were obtained by pretreatment with a ten-fold greater dose of indomethacin (14 µmol/100 g i.v.), an amount likely to inhibit prostaglandin dehydrogenase as well as prostaglandin synthetase (R.J. Flower, personal communication).

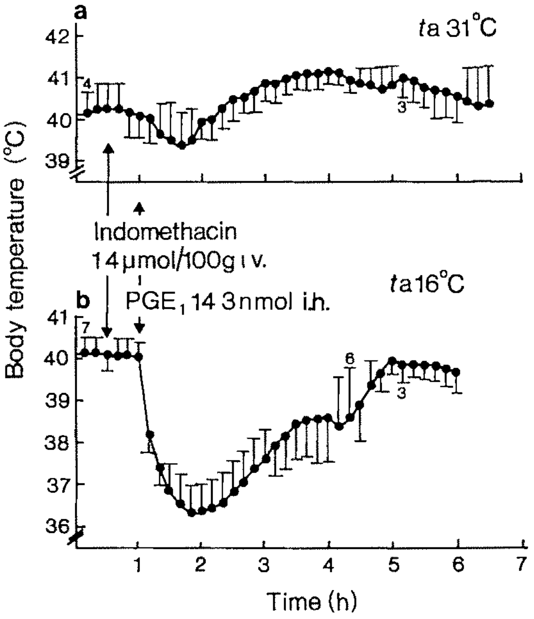

Figure 3 Mean effects (± s.e. mean) of intrahypothalamic injection (i.h.) of prostaglandin E_1 (PGE_1, 14.3 nmol) given 30 min after indomethacin (1.4 µmol/100 g i.v.) at an ambient temperature (t_a) of (a) 31° and (b) 16°C. Compare with Figure 1.

Effects of prostaglandin E_1 after 5,8,11,14-eicosatetraynoic acid (ETA)

The effects of prostaglandin E_1 were also intensified and prolonged after pretreatment with ETA (1 mg/100 g i.v. 30 min previously), a more selective inhibitor of prostaglandin synthetase than indomethacin. Thus at thermoneutrality (31°C) and after ETA pretreatment, body temperature of one chick rose 1.5°C after intrahypothalamic injection of prostaglandin E_1 (14.8 nmol), recovery not occurring until 8 h later. Similarly below thermoneutrality (16°C), the mean duration of the hypothermic effects of prostaglandin E_1 (14.3 nmol) in 4 chicks was significantly increased by ETA ($P < 0.05$) from 108 ± 20 min with prostaglandin E_1 alone to 312 ± 92 min (mean ± s.e. mean); the mean fall in temperature was 5.6°C, significantly greater ($P < 0.05$) than that after prostaglandin E_1 alone (2.45°C).

Effects of the 'O'-somatic antigen of Shigella dysenteriae

Within thermoneutrality (31°C) injections of the 'O'-somatic antigen of *Shigella dysenteriae* (0.1 and 1 µg) into the hypothalamus rapidly evoked pyrexia, that after 1 µg being the longer-lasting: effects of 10 ng

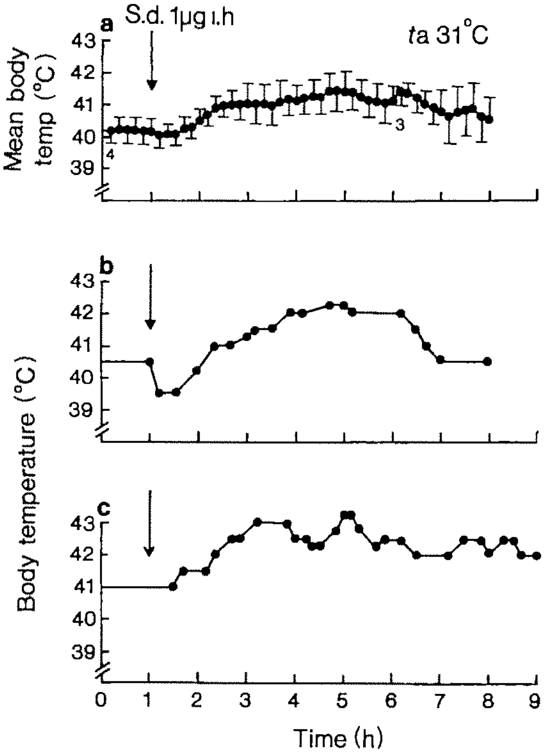

Figure 4 Mean effects (± s.e. mean) of *Shigella dysenteriae* (S.d.) on body temperature of four chicks at an ambient temperature (*ta*) of 31°C (a); (b) and (c) illustrate two types of effect observed in individual chicks.

were inconsistent. The mean pyrexic effect (4 chicks) of the 1 µg dose is illustrated in Figure 4a; body temperature was unaffected by intrahypothalamic injection of the control vehicle, it remaining within the limits observed during the control period. After a small reduction, mean body temperature began to increase after 60 min attaining a maximum increase of 1.2°C after the infusion, and was still 0.6°C above pre-infusion values 3 h later. This mean temperature response probably conceals two different forms of response to the endotoxin, one (2 chicks) in which a fall in body temperature preceded a sustained rise in body temperature (Figure 4b) and another, (2 chicks) in which body temperature increased within 30 min of infusion without a prior fall in body temperature (Figure 4c).

Below thermoneutrality (16°C), mean body temperature initially rose 0.95°C with the injection of 'O'-somatic antigen of *Shigella dysenteriae* (1 µg) then declined again to pre-injection values followed by a more sustained temperature increase of 1.2°C and returned to control values 12–13 h after injection. Again the mean pattern concealed apparently different

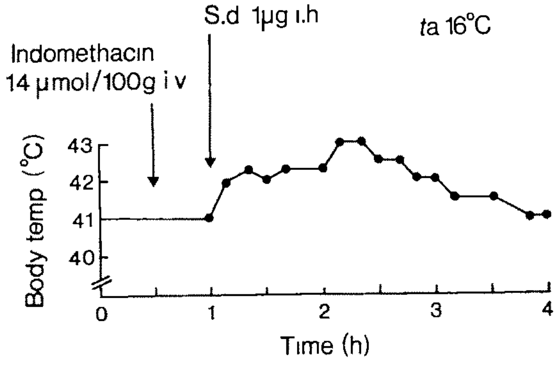

Figure 5 Effect of indomethacin (14 µmol/100 g i.v.) on the pyrexic response to *Shigella dysenteriae* (S.d., 1 µg injected into the hypothalamus, i.h., 30 min later). Ambient temperature (t_a), 16°C.

types of body temperature response to bacterial pyrogen. In 3 chicks the pattern conformed to that for the mean body temperature change, in 2 chicks there was a delay of up to 2 h before body temperature began to increase and in 1 chick, increase in body temperature commenced immediately after injection.

Effects of the 'O'-somatic antigen of Shigella dysenteriae *after indomethacin*

In each of three chicks tested within thermoneutrality (31°C), intrahypothalamic injection of the 'O'-somatic antigen of *Shigella dysenteriae* (1 µg) 30 min after injection of indomethacin (1.4 µmol/100 g i.v.) evoked hyperthermia. The mean increase in body temperature was the same as that seen after endotoxin alone (1.2°C). However, the duration of the response was reduced from more than 7 h to between 5 and 6.5 hours.

In 5 chicks tested below thermoneutrality (16°C), the 'O'-somatic antigen of *Shigella dysenteriae* (1 µg) elevated body temperature a mean of 1.5°C when given 30 min after indomethacin (1.4 µmol/100 g i.v.). Again the duration of response was attenuated, recovery occurring within 3.5 hours. Figure 5 illustrates persistence of the hyperthermic response to endotoxin even after a large dose of indomethacin (14 µmol/100 g, 30 min previously).

Antagonism of endotoxin pyrexia by prostaglandins E_1 and E_2 at 16°C

Injected into the hypothalamus at varying times after *Shigella dysenteriae*, prostaglandins E$_1$ and E$_2$ either prevented the onset of hyperthermia or reversed it. Thus in Figure 6a, prostaglandin E$_1$ (14.3 nmol) was injected 1.75 h after endotoxin (1 µg) when a rise in body temperature would be expected to occur; body temperature rapidly fell by 8.5°C within 1.5 h of infusion. Recovery approximately 6 h later was

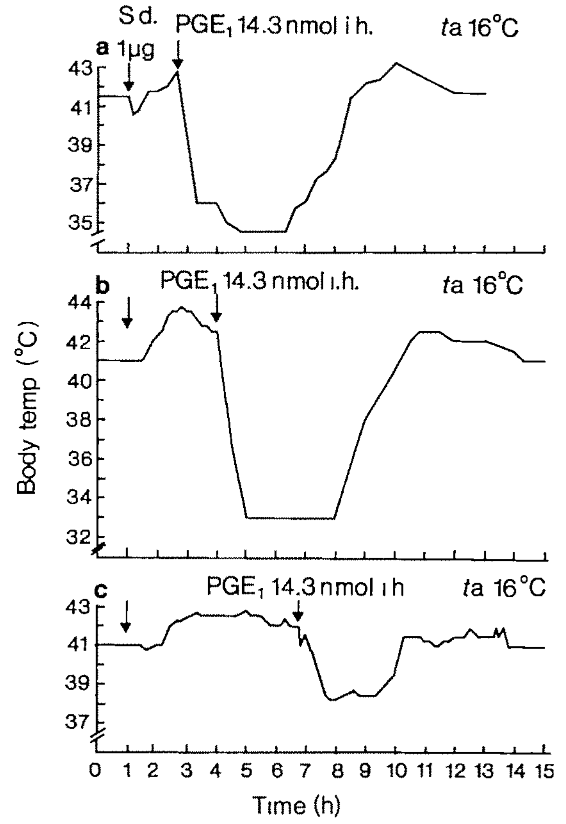

Figure 6 Effects of intrahypothalamic injection (i.h.) of prostaglandin E$_1$ (PGE$_1$, 14.3 nmol) at different intervals after intrahypothalamic injection of *Shigella dysenteriae* (S.d., 1 µg). Prostaglandin E$_1$ was given at the start (a), during (b), and towards the end (c) of the induced pyrexia. Ambient temperature (t_a), 16°C.

followed by an overshoot of 1.5°C before body temperature fiinally returned to normal. The hypothermic response to prostaglandin E$_1$ was apparently potentiated since the temperature fall of 8.5°C compares with a mean fall of 2.1°C after prostaglandin E$_1$ alone (Figure 1b). A similar potentiation was seen when prostaglandin E$_1$ was given shortly after onset of hyperthermia (Figure 6b). In this experiment, prostaglandin E$_1$ (14.3 nmol) was injected 3 h after the endotoxin at which time body temperature was elevated 1.5°C; prostaglandin E$_1$ lowered body temperature some 9.5°C, recovery again being followed by the characteristic overshoot. This enhanced response to prostaglandin E$_1$ was not seen when it was given in the later stages of endotoxin fever, 6 h after the pyrogen (Figure 6c). The hypothermic effects of prostaglandin E$_2$after the 'O'-somatic antigen of *Shigella dysenteriae* were similar to those of prostaglandin E$_1$.

Suppression of endotoxin pyrexia by noradrenaline, 5-hydroxytryptamine and carbachol at 16°C

Noradrenaline, 5-hydroxytryptamine and carbachol each produced hypothermia when given alone into the hypothalamus of chicks maintained at 16°C and each counteracted endotoxin pyrexia produced by intrahypothalamic infusion of the 'O'-somatic antigen of *Shigella dysenteriae* (1 µg).

Noradrenaline. When given during the early stages of endotoxin fever at a time when body temperature was elevated 0.75–1°C (2 chicks), noradrenaline (0.05 µmol) abolished the pyrexia and lowered body temperature, although its effects were considerably attenuated. In one chick, maximum fall in body temperature was 8.25°C with recovery after 4 h and in the other, 5.5°C with recovery 2.5 h later; these compare with a mean maximum fall of 15.7°C and recovery in 6–14 h after noradrenaline alone (Marley & Stephenson, 1975).

5-Hydroxytryptamine. 5-Hydroxytryptamine (0.05 µmol) injected into the hypothalamus (3 chicks) lowered body temperature irrespective of whether it was given at the onset or during endotoxin fever. Thus in one chick in which body temperature was elevated 1.25°C, 1.5 h after giving *Shigella dysenteriae*, 5-hydroxytryptamine lowered body temperature 4.25°C with recovery, followed by an overshoot of 0.8°C min later.

Carbachol. Carbachol lacked effect on body temperature when given alone at thermoneutrality (Marley & Seller, 1974) but when given below thermoneutrality it was found to be a potent hypothermic agent. Figure 7 illustrates reversal of

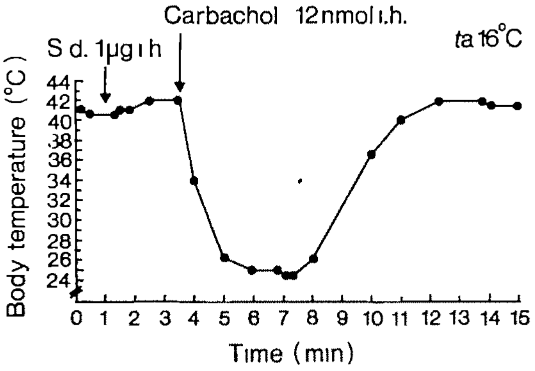

Figure 7 Reversal of endotoxin fever, induced by intrahypothalamic infusion of *Shigella dysenteriae* (S.d., 1 µg), by intrahypothalamic injection (i.h.) of carbachol (12 nmol). Ambient temperature (t_a), 16°C.

established endotoxin hyperthermia by carbachol (12 nmol) injected into the hypothalamus.

Discussion

In young chicks at thermoneutrality, prostaglandin E$_1$ and E$_2$ injected into the hypothalamus elevated body temperature, an effect similar to that seen after their intraventricular or intrahypothalamic injection in adult fowls (Nisticò & Marley, 1973; Kane & Peterson, 1975). However, in chicks maintained at an ambient temperature below thermoneutrality (16°C), intrahypothalamic injections of prostaglandin E$_1$ and E$_2$ lowered body temperature. The potencies of E$_1$ and E$_2$ prostaglandins were similar, E$_1$ possibly being slightly more active than E$_2$ at thermoneutrality, whereas below thermoneutrality the converse was true. It is unlikely that the hypothermic responses to prostaglandin E$_1$ could be attributed to a general proclivity of compounds affecting thermoregulation to depress the body temperature in young chicks when administered below thermoneutrality, since under these circumstances it might be expected that the 'O'-somatic antigen of *Shigella dysenteriae* would also be hypothermic. An ambient temperature of 16°C is not a severe cold stress for young chicks of this age, since plasma non-esterified fatty acids were not significantly elevated and the chicks were able to maintain normal body temperature for over 48 h without shivering (Marley & Stephenson, 1975).

Pretreatment with indomethacin potentiated the hypothermic responses to prostaglandin E$_1$. Assuming indomethacin to be an effective inhibitor of prostaglandin synthetase then the results could be explained by the presence of two prostaglandins with opposite effects on body temperature, an increased effect of one being counterbalanced by increased release of the other. When the synthesis of both prostaglandins is inhibited, the injected prostaglandin will exert its effect unopposed by the endogenous prostaglandin with the opposite effect. A similar explanation for potentiation of the opposing effects on bronchial tone of prostaglandin E$_1$ and prostaglandin F$_{2\alpha}$ after indomethacin has been advanced by Frey & Schafer (1974). A simpler alternative explanation, that indomethacin was also inhibiting prostaglandin 15-dehydrogenase, is less likely, since the effects of prostaglandin E$_1$ were also potentiated by pretreatment with ETA, a more selective inhibitor of prostaglandin synthetase. Should the above explanation be correct then the other prostaglandin is not prostaglandin F$_{2\alpha}$ since this is hypothermic at all ambient temperatures studied, both above and below thermoneutrality (Whelan, 1976).

The opposite effects on body temperature of E series prostaglandins, depending on ambient temperature, and indeed the antagonism below thermoneutrality of endotoxin fever by prostaglandins E$_1$ and E$_2$, do not necessarily preclude these as mediators of endotoxin fever in chicks. There remains the possibility that hypothalamic prostaglandins, in addition to their role in the genesis of fever, are also involved in the regulation of peripheral vasomotor tone, the actions of prostaglandin E$_1$ and E$_2$ being to cause vasodilatation. Such an action, while not preventing hyperthermia induced by intrahypothalmic infusion of prostaglandins E$_1$ and E$_2$ at thermoneutrality, may reverse their hyperthermic action when given below thermoneutrality (not unreasonable considering the large surface/volume ratio of young chicks) and be sufficient to overcome pyrogen fever evoked by a more localized release of endogenous prostaglandin E$_1$ in response to administration of the 'O'-somatic antigen of *Shigella dysenteria*. However, hypothermic responses to prostaglandins of the E series have also been reported in the adult spiny anteater, *Tachyglossus aculeatus* (Baird *et al.*, 1974). In this non-placental mammal, intraventricular prostaglandins E$_1$ and E$_2$ lowered body temperature in both warm and cool environments and, as in the chick, hypothermia was associated with peripheral vasodilatation. The reduction in metabolic rate observed in both species may have been secondary to behavioural sedation in young chicks and to the 'general relaxation' observed in the anteater. It would be of considerable interest to know whether or not prostaglandins E$_1$ and E$_2$ were also hypothermic after injection into the preoptic/anterior hypothalamic area of the anteater since this species (a monotrematous mammal) has, unlike birds, evolved from the same reptilian stock as the placental mammals. Below thermoneutrality (16°C), endotoxin fever was also antagonized by intrahypothalamic infusions of noradrenaline, 5-hydroxytryptamine and carbachol, compounds which produce hypothermia when given alone. Interestingly, carbachol lacked effect on body temperature when given at thermoneutrality (Marley & Seller, 1974).

Failure of indomethacin and ETA to prevent onset of endotoxin fever and to abolish established fever make it uncertain that prostaglandins mediate endotoxin fever in chicks. However such a conclusion can only be tentative until the significance of the moderate reduction in the duration of the hyperthermic response to endotoxin after indomethacin and ETA is known and also the degree to which indomethacin and ETA inhibited prostaglandin synthesis. Experiments are in progress to answer these questions.

In summary, the present experiments demonstrate that below thermoneutrality, prostaglandins of the E series produce hypothermia when infused into the hypothalamus of young chicks and that the hypothermia is sufficient to antagonize endotoxin fever. Endotoxin fever is not prevented or abolished by two inhibitors of prostaglandin synthetase, indomethacin and ETA. Doses of prostaglandin and endotoxin are larger than those used in many mammalian studies but this probably relates to the known resistance of birds to pyrexic agents (Sollman, 1957) and to endotoxin (Jordan & Hinshaw, 1964). Experiments are now in progress to determine whether or not prostaglandins of the E series can be excluded as mediators of endotoxin fever in chicks.

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(Received October 19, 1976.
Revised April 7, 1977.)

Br. J. Pharmac. (1977), **61**, 47–55

PHARMACOLOGICAL AND CERTAIN CHEMICAL PROPERTIES OF AH 10407, AN UNUSUALLY SHORT-ACTING, COMPETITIVE NEUROMUSCULAR BLOCKING DRUG, AND SOME RELATED COMPOUNDS

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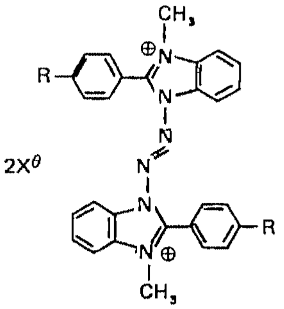
1 1,1′-Azobis[3-methyl-2-phenylbenzimidazolinium]dimethanesulphonate (AH 10407) has an ultra-short, competitive neuromuscular blocking action in the mouse, cat, dog, Cynamolgus monkey and cotton-eared marmoset.

2 AH 10407 is chemically unstable in bicarbonate-containing solutions and is degraded to inactive products. The half-life of AH 10407 *in vitro* in dog and human whole blood and in Krebs physiological solution is about 1.0 minute. In distilled water and in HCO_3^--deficient Krebs solution AH 10407 is much more stable. Base catalyzed degradation is shown to be the prime determinant of the duration of action of the drug.

3 Some pharmacological properties of AH 11244 and AH 11056, close analogues of AH 10407, are briefly described and the duration of their neuromuscular blocking actions rationalized by reference to their chemical stabilities.

Introduction

Many compounds expected to be easily susceptible to enzymatic degradation have been made in attempts to find a competitive neuromuscular blocking (NMB) drug with a short duration of action in man (Bruce, 1956; Haining, Johnston & Smith, 1960; Brittain, Collier & D'Arcy, 1961; Savarese, Nakamura & Kitz, 1970; Savarese, Ginsburg, Lee & Kitz, 1973) but only succinylcholine, a depolarizing agent, has proved satisfactory in this respect. An alternative, novel approach to the desired short-acting, competitive NMB drug in man was identified during the pharmacological and physico-chemical examination of 1,1′-azobis[3-methyl-2-phenylbenzimidazolinium] dimethanesulphonate (AH 10407; Blogg, Brittain, Simpson & Tyers, 1975; Tyers, 1975), one of a series of azobis-benzimidazolinium salts (Glover, Bishop & Rowbottom, 1973). AH 10407 (1a) is a competitive, neuromuscular blocking drug with an ultra-short duration of action in animals and in man. This paper describes the detailed pharmacology of AH 10407 in laboratory animals, including primates, and describes experiments undertaken to account for its brief action. A preliminary report on AH 10407 has been presented to the British Pharmacological Society (Blogg *et al.*, 1975). Limited pharmacological results are also presented on AH 11244 [1b] and AH 11056 [1c] which are close analogues of 10407.



1a AH 10407 R=H
1b. AH 11244 R=CH_3
1c. AH 11056 R=OCH_3

Methods

Paralysing action in chicks

The paralysing activity of intravenous AH 10407 was determined in 7-day-old chicks. Groups of 6 chicks were used for each dose-level. The ED_{50} (paralysing activity) for AH10407 was calculated by the method of Litchfield & Wilcoxen (1949).

Neuromuscular blocking actions in anaesthetized cats, dogs and monkeys

Cats (1.7–3.2 kg) were anaesthetized with chloralose (75 mg/kg intravenously) following induction with a mixture of halothane (3%) in nitrous oxide and oxygen (3 : 1). The tibialis anterior muscle was prepared for indirect stimulation via the peroneal branch of the sciatic nerve by the method described previously (Brittain & Tyers, 1973). The peroneal nerve was stimulated at a frequency of 1 Hz with rectangular pulses of supramaximal voltage and 0.2 ms duration. Tibialis anterior muscle twitches were recorded on an Elema-Schonander Mingograf recorder. The rate of onset, the intensity, the rate of recovery and the duration of the neuromuscular blocking actions of AH 10407 were determined from the following measurements of tibialis anterior muscle twitches; (a) the time from intravenous injection to the first reduction in twitch tension; (b) the time from the first reduction in twitch tension to maximum neuromuscular block; (c) the maximum percentage inhibition of muscle twitches; (d) the time from the first reduction to 50% recovery of the twitch height; (e) the time from first injection to 90% recovery of the twitch height. In some experiments the tibialis anterior muscle was stimulated directly by inserting a concentric, bipolar, stainless-steel electrode into the belly of the muscle. Close-arterial injections of drugs to the tibialis anterior muscle were carried out via the cannulated suralis branch of the popliteal artery; the femoral artery was occluded during close-arterial injections. Rectal temperature was monitored throughout. Where relevant, tibialis anterior muscle temperatures were measured with thermocouple probes inserted deep into the belly of the muscle.

Similar experiments were carried out in Beagle dogs (7.9–8.5 kg) Cynamolgus monkeys (1.6–2.3 kg) and cotton-eared marmosets (0.5–0.7 kg). The dogs and primates were anaesthetized with pentobarbitone (25–35 mg/kg i.v.) to effect and maintain surgical anaesthesia.

Neuromuscular blocking actions on the isolated phrenic nerve-diaphragm preparation of the rat

Neuromuscular blocking activity was determined on the isolated phrenic nerve-diaphragm preparation of the rat according to the method described by Brittain & Tyers (1973). The diaphragm was stimulated indirectly with trains of pulses (45 Hz for 0.2 s and 0.2 ms pulse width) of supramaximal intensity given once every 15 seconds. Neuromuscular blocking activities (EC_{50}) were calculated from cumulative dose-response curves.

Actions on the cardiovascular system in anaesthetized cats, dogs and monkeys

The effects of single doses or continuous intravenous infusions of AH 10407 on the cardiovascular system were investigated in the anaesthetized cats, dogs, monkeys and marmosets used in the neuromuscular studies. The carotid blood pressure, pulse rate and the electrocardiogram (lead II) were recorded in all experiments. In the anaesthetized cat the effects of AH 10407 on the blood pressure and pulse rate responses induced by peripheral vagal nerve stimulation were also recorded.

Actions on ganglionic transmission

In cats anaesthetized with chloralose the effects of AH 10407 on contractions of the nictitating membrane to periodic, preganglionic stimulation for 6 s of the ascending cervical sympathetic nerve (6 Hz, 0.5 ms pulse width, supramaximal intensity) were measured with a 2 oz strain guage transducer and recorded on the chart recorder. In dogs anaesthetized with pentobarbitone the effects of AH 10407 on vasopressor responses to injected dimethylphenylpiperazinium iodide (DMPP) were also determined.

Histamine release

The histamine-releasing activity of AH 10407 was investigated in two species. In guinea-pigs anaesthetized with urethane the bronchoconstrictor test described by Dixon & Brodie (1903) was used. In anaesthetized cats the effects of mepyramine on the delayed vasodepressor response described by Collier & Macauley (1952) were studied.

Chemical stability

In early experiments (Tyers, 1975) it was found that the neuromuscular blocking action of AH 10407 on the rat isolated phrenic nerve-diaphragm preparation waned during 2 min continuous exposure to drug-containing Krebs solution. This result, together with the finding of Glover *et al.* (1973) that AH 10407 and some of its analogues were unstable in water in the presence of nucleophiles such as OH^- or HCO_3^- led us to a more systematic investigation of the stability of AH 10407 under a variety of conditions because base-catalyzed inactivation in blood seemed a possible determinant of its duration of action. Solutions of the drug in distilled water, aqueous solution adjusted to pH 1, 3 or 6 with hydrochloric acid, whole blood (dog and human), normal Krebs solution, Krebs solution without sodium bicarbonate and Krebs solution containing an equivalent amount of sodium acetate instead of bicarbonate were assayed after various periods of incubation at 24–40°C on the rat isolated phrenic

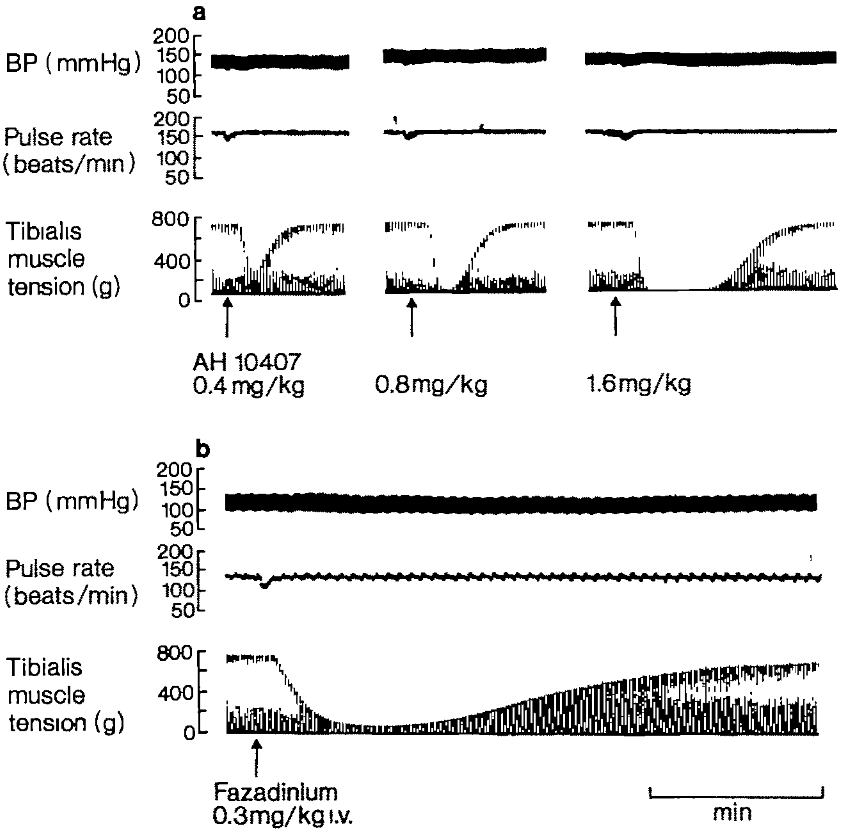


Figure 1 The effects of intravenous doses of (a) AH 10407 and (b) fazadinium dibromide (AH 8165D) on tibialis anterior muscle twitches stimulated indirectly at a frequency of 1 Hz, on arterial blood pressure (BP) and pulse rate in the cat anaesthetized with chloralose.

nerve-diaphragm preparation with the freshly prepared aqueous drug solution as standard.

Drugs and solutions

The following drugs were used: 1,1′-azobis[3-methyl-2-phenylbenzimidazolinium] dimethanesulphonate (AH 10407); 1,1′-azobis[3-methyl-2-*p*-tolyl-benzimidazolinium]dibromide (AH 11244); 1,1′-azobis[3-methyl-2-*p*-methoxyphenyl-benzimidazo-linium]dibromide (AH 11056); fazadinium dibromide (AH 8165, Allen & Hanburys Research Ltd.); (+)-tubocurarine chloride (Burroughs Wellcome); neostigmine methylsulphate (Roche Products Ltd.); atropine sulphate (Macfarlane Smith Ltd.); acetylcholine chloride (Sigma); dimethylphenylpiperazinium iodide. Drugs were dissolved and diluted in 0.9% w/v NaCl solution (saline). All doses and concentrations refer to the free bases. In anaesthetized preparations all drugs were given intravenously unless stated otherwise.

Results

Paralysing action in chicks

In conscious, 7-day-old chicks AH 10407, 0.8 mg/kg intravenously, caused a flaccid paralysis which lasted for 25 seconds. The ED_{50} (paralysing activity) values for AH 10407 and (+)-tubocurarine were 0.42 (0.28–0.60) mg/kg and 0.045 (0.035–0.05) mg/kg respectively.

Neuromuscular blocking actions in anaesthetized cats, dogs, Cynamolgus monkeys and marmosets

In the anaesthetized cat, AH 10407 0.5–2.0 mg/kg intravenously, depressed tibialis anterior muscle twitches (frequency, 1 Hz) by 14.3–100%. At these doses the neuromuscular block lasted from 6–50 seconds. The onset of block induced by AH 10407 was very rapid, the maximal effects being reached within 10–15 s after injection. The neuromuscular blocking actions of AH 10407 and fazadinium in the cat are compared in Figure 1. AH 10407 had similar effects in the other species studied (Table 1). The species variation in sensitivity to AH 10407 was slight. The onset times of block were similar in the cat and Cynamolgus monkey, but were slightly longer in the dog and marmoset. AH 10407 was longer acting in the marmoset than in the cat, dog or Cynamolgus monkey, but even in this species, which has been found to resemble man closely regarding the duration of action of some competitive NMB drugs (Tyers, 1975), near-complete neuromuscular block lasted for only 3 minutes. In the anaesthetized cat the 'cumulation time' for repeated submaximal doses of AH 10407 was 1.5 min, indicating that full recovery of neuromuscular transmission was very rapid.

Intravenous infusions of AH 10407, 0.45 mg kg^{-1} min^{-1}, in the anaesthetized cat and Cynamolgus monkey caused 80–95% inhibition of twitches of the tibialis anterior muscle. When the infusions were terminated after 30 min, recovery from the neuromuscular block was complete within 30 seconds.

Variations in muscle temperature affected the neuromuscular blocking action of AH 10407. In the anaesthetized cat its potency and duration of action were greater in cooled muscle than in warmed muscle (Figure 2). In contrast, fazadinium and (+)-tubocurarine were more active and longer acting on the warmer muscle (Table 2).

Apnoea induced by AH 10407 in the cat was only seen with doses 3–4 times those required to inhibit tibialis anterior muscle twitches; for example, in one preparation 2 mg/kg caused 88% blockade of muscle twitches, but 6 mg/kg was required to arrest respiration. At this higher dose, muscle twitches were blocked for 87 s while respiration was arrested for only 12 seconds.

Table 1 Neuromuscular blocking potencies and time course of action of AH 10407 in the anaesthetized cat, dog, monkey and marmoset following intravenous injection

		Inhibition of tibialis muscle twitches (frequency, 1 Hz)				
					Recover times (min:s)	
Species (number of animals)	*Dose mg/kg (i.v.)*	*Time from injection to first detectable actions (s)*	*Time from detectable action to maximum effect (s)*	*Mean block (%)*	*50%*	*90%*
Cat (5)	0.5	6	3	14.3	0:04	0:06
		(4–7)	(2–5)	(0–22 4)	(0–0:06)	(0–0.10)
	1.0	4.5	6	85.9	0:12	0:17
		(3–6)	(5–7)	(83.3–88.4)	(0:08–0:15)	(0:12–0:25)
	2.0	5	5.5	100	0:28	0:35
			(5–6)		(0:23–0:34)	(0:29–0:50)
Dog (2)	1.0	5	8	34.2	0:08	0:12
		(4–6)	(5–9)	(25.2–40.0)	(0:02–0:16)	(0:05–0:20)
	2.0	5	7.5	73.8	0:14	0:17
		(4–6)	(5–9)	(68.2–81.4)	(0:08–0:21)	(0·10–0:24)
	4.0	5	14	97 5	0:52	1:02
		(4–6)	(9–15)	(85.0–100)	(0:45–0·59)	(0:49–1:12)
Cynamolgus monkey (2)	0.5	4	4	4.0	0:04	0:06
		(3–5)	(3–5)	(0–8.0)	(0–0:06)	(0–0:10)
	1.0	4	7	67.0	0:15	0·19
		(3–5)	(5–8)	(48.0–73.0)	(0:09–0:20)	(0:13–0:23)
	2.0	5	5	95.0	0:45	0:51
		(3–7)	(4–6)	(89.2–100)	(0:35–0:50)	(0:38–1:00)
Cotton-eared marmoset (1)	1.0	5	14	50.9	0:26	0:47
		(4–6)	(9–16)	(44.5–57.4)	(0:09–0:43)	(0:14–1:20)
	2.0	5	10	95.4	1·51	3:05
		(4–6)	(8–13)	(94.4–96.4)	(1:02–2:20)	(2:59–3:12)

Values in the table refer to means (range) of responses.

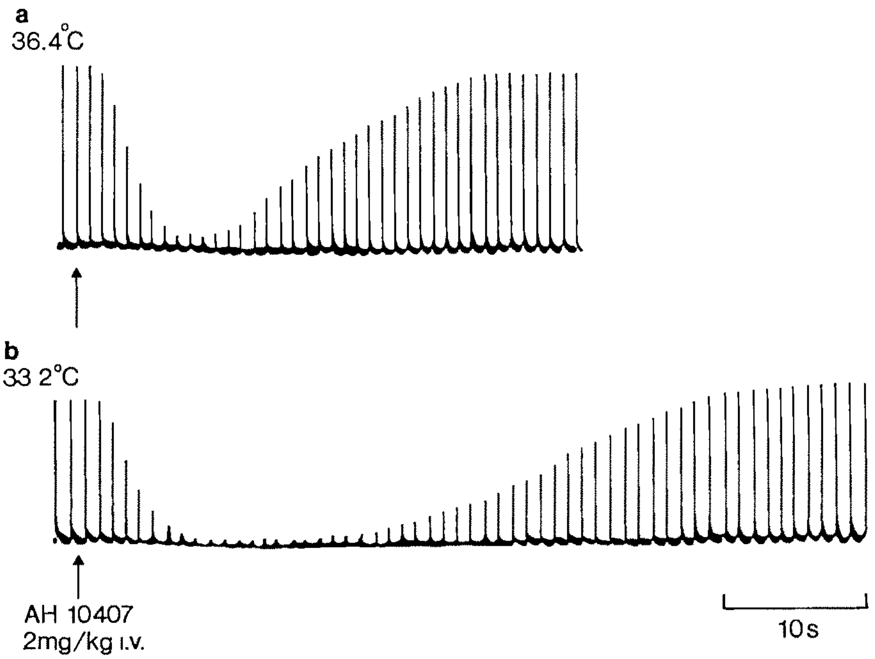


Figure 2 The effects of lowered muscle temperature on the neuromuscular blocking actions of AH 10407 in the cat anaesthetized with chloralose. The effects of an intravenous dose of AH 10407 on indirectly evoked twitches of the right tibialis anterior muscle (a) which was at 36.4°C and the left tibialis anterior muscle (b) which was cooled to 33.2°C, by packing the leg in ice, are compared in the same cat. Muscle temperature was measured with a deep muscle thermocouple probe.

Site and mechanism of action. The neuromuscular block induced by AH 10407 was primarily due to an action of the motor end-plate since doses of 0.5–4.0 mg/kg blocked twitches of the tibialis anterior muscle evoked by close-arterial injections of acetylcholine but not those evoked by direct electrical stimulation. The blockade was neither preceded by potentiation of the muscle twitch response nor associated with muscle fasciculations. Close-arterial injections of AH 10407, 100 µg, to the tibalis anterior muscle reduced indirectly evoked twitches and did not cause a contracture. The neuromuscular block induced by an intravenous infusion of AH 10407, 0.5 mg kg^{-1} min^{-1}, was rapidly and completely reversed by neostigmine. During partial block with AH 10407, muscle responses to tetanic stimulation

Table 2 The effects of muscle temperature on the neuromuscular blocking actions of fazadinium and (+)-tubocurarine in the anaesthetized cat

		Neuromuscular block	
Drug and dose (mg/kg i.v.)	*Muscle temp. (°C)*	*% Inhibition of tibialis muscle twitches*	*Duration of action (min)*
Fazadinium	31.5	41.3 ± 8.4	1 37 ± 0.33
(0.2 mg/kg)	36.0	64.9 ± 9.7	1.65 ± 0.53
	39.5	79.1 ± 10.7	2.10 ± 0.44
(+)-Tubocurarine	31.5	49.7 ± 11.1	10.2 ± 4.2
(0.2 mg/kg)	36.0	82.4 ± 12 7	12.4 ± 5.7
	39.5	100	16.7 ± 3.5

Results are means ± s.e. ($n = 4$)

were poorly sustained and there was a post-tetanic facilitation of the muscle twitches. No changes in the tibialis muscle surface potential occurred during AH 10407 blockade. The effects of competitive neuromuscular blocking drugs given concurrently with AH 10407 were additive, whereas depolarizing neuromuscular drugs antagonized the actions of AH 10407. All these characteristics show AH 10407 to be a competitive neuromuscular blocking drug.

Actions on the cardiovascular system in anaesthetized cats, dogs, Cynamolgus monkeys and marmosets

In artificially ventilated, anaesthetized cats neuromuscular blocking doses of AH 10407, 0.5–4.0 mg/kg intravenously, caused slight falls in mean arterial blood pressure (5–30 mmHg); 10 mg/kg caused a fall of 50 mmHg. These doses had no effect on the heart rate or ECG. The vasodepressor responses caused by AH 10407 lasted only 2–3 min but were slightly more persistent than the neuromuscular block. In artificially ventilated dogs, Cynamolgus monkeys and cotton-eared marmosets, single doses of AH 10407, 0.5–4 mg/kg intravenously, caused only minimal falls in blood pressure (5–15 mmHg). Prolonged intravenous infusions of 0.5 mg kg^{-1} min^{-1} in the anaesthetized cat reduced the blood pressure by 35 ± 14 mmHg and the heart rate by 25 ± 10 beats/minute.

In the anaesthetized cat, AH 10407, 0.5–2.0 mg/kg, caused dose-dependent inhibition (8–75%) of the hypotensive and negative chronotropic effects caused by periodic preganglionic stimulation of the vagus nerve. The inhibitory effect lasted for less than 60 seconds.

Effects on ganglionic transmission

In the anaesthetized cat the vasodepressor responses caused by AH 10407, 0.5–4.0 mg/kg intravenously, were accompanied by dose-dependent inhibition (20–50%) of contractions of the nictitating membrane evoked by periodic preganglionic sympathetic nerve stimulation; responses of the nictitating membrane to post-ganglionic stimulation of the cervical sympathetic nerve were unaffected.

In the dog anaesthetized with pentobarbitone, AH 10407, 2 and 10 mg/kg intravenously, inhibited vasopressor responses caused by DMPP 0.1 µg/kg intravenously, by 10 and 75% respectively. When these large doses of AH 10407 were repeated at intervals of less than 6 min a cumulative ganglion blocking action was seen which lasted for longer than the neuromuscular blockade.

Histamine release

In guinea-pigs anaesthetized with urethane AH 10407, 0.5–10 mg/kg intravenously, did not increase bronchial resistance whereas a substantial increase in bronchial resistance was seen after (+)-tubocurarine 0.2 mg/kg intravenously. Furthermore, in cats anaesthetized with chloralose, pretreatment with mepyramine 1 mg/kg, did not modify the vasodepressor responses induced by AH 10407. The results show that AH 10407 does not release histamine.

Neuromuscular blocking action on the isolated phrenic nerve-diaphragm preparation of the rat

AH 10407 inhibited diaphragm muscle twitches evoked by phrenic nerve stimulation. The EC_{50} (±s.e.) value for AH 10407 on this preparation was 9.5 ± 1.4 µg/ml. The course of investigation into the reasons for the termination of the neuromuscular blocking action of AH 10407 stemmed from the observation that the blocking effect of AH 10407 on the rat isolated phrenic nerve-diaphragm preparation terminated before the drug had been washed from the bathing fluid. Studies were therefore carried out to determine the chemical stability of AH 10407.

Chemical stability

AH 10407, dissolved in distilled water or bicarbonate-free Krebs solution, did not significantly decompose within 60 min at 37°C, but was unstable in Krebs solution and in dog and human whole blood, the half-life being about 1 to 2 minutes. The compound was somewhat more stable in Krebs solution containing sodium acetate which is less basic than sodium bicarbonate. These results are summarized in Figure 3. The rate of degradation of AH 10407 was, as expected, more rapid at higher than at lower incubation temperatures. For example, at temperatures of 24, 32 and 40°C AH 10407 was completely degraded in Krebs solution within 15, 5 and 1 min respectively.

It is clear from the results in Table 3 that AH 10407 was more stable in the more acidic solutions.

Analogues of AH 10407

The results given in the previous section show that AH 10407 is very susceptible to base-catalyzed degradation. A possible reaction mechanism, proposed by our colleagues, Dr J. Clitheroe and M. Wadsworth, is shown in Figure 4. The nucleophilic attack occurs at the electron deficient C_2 position and this results in opening of the imidazole ring and cleavage of the tetrazene chain (indicated by the broken lines a and b respectively). The proposed end-products are 3-methyl-2-phenyl-benzimidazole (2) and the azidobenzanilide (3).

If the mechanism in Figure 4 were correct, more stable compounds would be attained by reducing the electron deficit at C_2, perhaps by introducing electron-

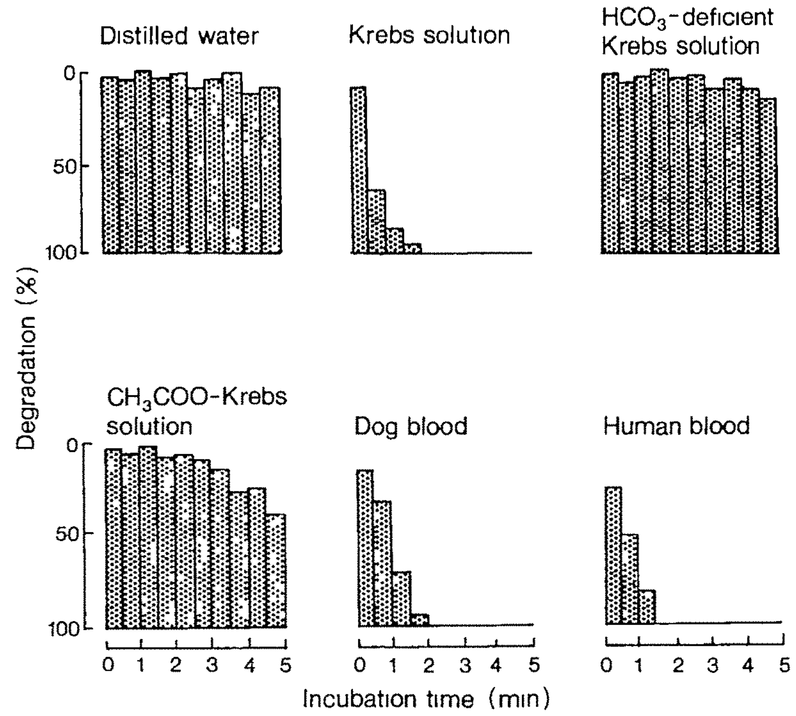


Figure 3 Rates of degradation of AH 10407 incubated *in vitro* at 37°C in various aqueous media. In Krebs physiological solution, dog blood and human blood AH 10407 was completely degraded within 2 minutes. In distilled water, Krebs solution deficient in HCO_3^- ions and in sodium acetate solution, AH 10407 was degraded much more slowly. The AH 10407 content in the incubates was assayed on the isolated phrenic nerve-diaphragm preparation of the rat

donating groups at the corresponding *para*-position in the 2-phenyl substituent. Such compounds would be expected to be longer-acting than AH 10407 and, unlike that compound, might be stable enough to be used clinically; for the latter purpose they would also, of course, have to be selectively active at the motor end-plate.

As a result of this analysis, AH 11244 and AH 11056, the *p*-tolyl and *p*-methoxyphenyl analogues, were made and tested for stability in Krebs solution and neuromuscular blocking activity in the

Table 3 The effect of pH on the stability of AH 10407 in distilled water at 22°C

Incubation time (h)	*Degradation of AH 10407 (%)*		
	pH 6	*pH 3*	*pH 1*
1	0	0	0
3	0	0	0
5	0	0	0
7	26.9	0	0
24	75.7	8.0	0
48	100	100	0
168	—	100.0	0

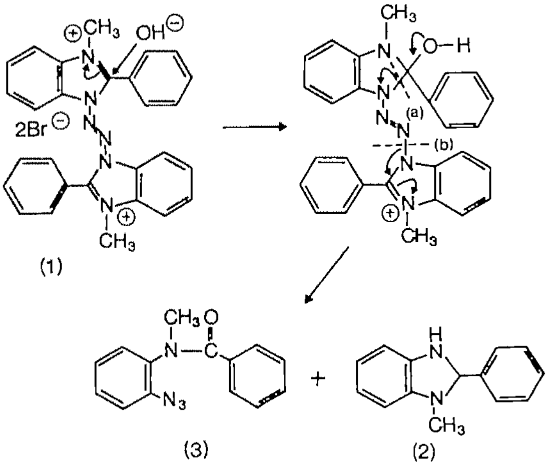


Figure 4 Proposed degradation pathway of AH 10407. The site of nucleophilic attack is the electron deficient C_2 position on one side of the molecule (1). The imidazole ring on that side opens (a) and there is a cleavage of the tetrazene chain (b) leaving the tertiary base (2) and the azidobenzanilide (3).

Table 4 Comparison of the rates of degradation of AH 10407, AH 11244 and AH 11056 incubated in normal Krebs solution at 37°C and their neuromuscular blocking potencies and durations of action in the anaesthetized cat

		Inhibition of tibialis anterior muscle twitches (1 Hz) in the anaesthetized cat	
AH No.	*Half-life in Krebs solution (min)*	*ED_{50} (mg/kg i.v.)*	*Duration of action (s)*
10407	1.9	0.7	12
11244	2.3	0.8	16
11056	4.2	1.8	60

anaesthetized cat by methods already described. The results, summarized in Table 4, show that AH 11056 especially was more stable and longer-acting than AH 10407, results that are consistent with the starting hypothesis, since the methoxy group is a better electron donor than methyl. However, paralysing doses of AH 11056 were associated with marked falls in blood pressure in the anaesthetized cat, presumably due to ganglionic blockade, and so work on it was abandoned.

Discussion

1,1′-Azobis[3-methyl-2-phenylbenzimidazolinium]dimethanesulphonate, AH 10407, is a competitive neuromuscular blocking drug in the mouse, rat, cat, dog, Cynamologous monkey and cotton-eared marmoset. The drug has a rapid onset of action, but the most striking difference between this and other competitive neuromuscular blocking drugs is that its duration of action is extremely short. The most likely reason for this extremely short duration of neuromuscular block is that AH 10407 is chemically unstable in the presence of basic ions. Certainly the drug is rapidly degraded in physiological solutions and whole blood, yet it is relatively stable in distilled water and solutions which do not contain strongly basic ions. The rate of degradation of AH 10407 is also dependent upon the incubation temperature which may be the reason why the duration of neuromuscular block produced by AH 10407, unlike other competitive neuromuscular blocking drugs, is longer in cooled than in normothermic or warmed muscles. Furthermore, some analogues of AH 10407 which contain electron donating groups in the *para*-position of the phenyl rings are chemically more stable than AH 10407 and produce longer-lasting neuromuscular blockade in the anaesthetized cat.

Differences in the durations of action of AH 10407 in different species are more difficult to explain in terms of chemical instability. It is well known that competitive neuromuscular blocking drugs are longer-acting in primates than in lower animal species (Mushin & Mapleson, 1964; Hughes, 1972; Brittain & Tyers, 1972, 1973; Tyers, 1975). The same is also true for AH 10407 which is about five times longer-acting in the marmoset than in the cat and dog. A preliminary study in man (Blogg *et al.*, 1975) showed that the duration of action of AH 10407 would be similar to that found in the anaesthetized marmoset. It may be expected therefore that AH 10407 would be degraded at different rates in the different species. Surprisingly though, the degradation rates of AH 10407 in dog and human blood *in vitro* were very similar. It is clear therefore, that the rate of plasma clearance of AH 10407 cannot account for the termination of its neuromuscular blocking action. Furthermore, the very fast onset time to block for AH 10407 demonstrates that this drug can diffuse freely and rapidly across capillary barriers. It is unlikely therefore that the termination of action is limited by diffusional delays in clearing the drug from the extracellular fluid environment of the motor end-plate. It is postulated therefore that the rate of termination of action of AH 10407 is limited by the rate of dissociation of the drug from the acetylcholine receptors. However, the rate of chemical degradation also influences the duration of action of AH 10407 and its close analogues, AH 11056 and AH 11244, and thus it is tempting to propose that AH 10407 is degraded to inactive products whilst it is still bound to the acetylcholine receptors. The inactive degradation products would of course have to dissociate much more easily from the receptor than the parent molecule. The longer durations of action of AH 10407 in primates may then arise in two ways. First, the energy of activation for the dissociation of AH 10407 from the receptor is greater in primates than in lower species. This suggests that drug-receptor interactions differ qualitatively in different species and could involve exoreceptor sites in the binding of AH 10407. The increased binding energy may also reduce the susceptibility of bound AH 10407 to chemical degradation. An alternative, or perhaps complementary, explanation is that the amount of acetylcholine released from motor nerve endings is lower in primates than in the other species, since increased acetylcholine levels reduce or terminate the action of competitive neuromuscular blocking drugs on the motor end-plate. It is clear that to investigate these proposals it would be necessary to compare the reaction kinetics for the dissociation of AH 10407 from the receptor and for its degradation process.

On a broader basis, this hypothesis may also explain the species variations in the duration of action of other more stable, bis-quaternary competitive neuromuscular blocking drugs. For example, the rates of plasma clearance of the new neuromuscular blocking drug, fazadinium dibromide (AH 8165D) in the cat and man are very similar (Tyers, 1975), yet there is a considerable difference in the duration of neuromuscular blockade induced by fazadinium in these species.

AH 10407 has clinically desirable properties but unfortunately, its inherent instability caused insuperable problems in its chemical development and pharmaceutical formulation. Nevertheless, base-catalyzed degradation is a potentially useful, novel, deactivating mechanism which may yet prove to be useful in the development of a competitive neuromuscular blocking drug that is genuinely short-acting in man.

We wish to thank Mrs S. Trower, Mr S. Manning and Mr A. Cornish for technical assistance.

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Received November 3, 1976.
Revised February 17, 1977.)

Br. J. Pharmac. (1977), **61**, 57–63

THE DEPENDENCE OF THE LIPID BILAYER MEMBRANE: BUFFER PARTITION COEFFICIENT OF PENTOBARBITONE ON pH AND LIPID COMPOSITION

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1 The membrane/buffer partition coefficient of [^{14}C]-pentobarbitone has been determined as a function of the lipid composition of bilayer membranes.

2 A new technique based on ultrafiltration gave comparable results to conventional techniques but required less time for equilibration.

3 The membrane/buffer coefficient was independent of pentobarbitone concentration in the range studied.

4 The apparent partition coefficient varied with pH and was a linear function of the degree of dissociation of pentobarbitone.

5 Both the charged and uncharged forms of pentobarbitone partitioned into the membrane, the latter to a much greater extent than the former.

6 At low pH the highest partition coefficient observed was in egg phosphatidylcholine bilayer membranes.

7 Incorporation of cholesterol or phosphatidic acid into phosphatidylcholine membranes greatly reduced the partition coefficient.

8 High pressures do not greatly change these partition coefficients.

Introduction

The lipid solubility of barbiturates is one of the important controlling factors of their pharmacodynamics (Brodie, Kurz & Schanker, 1960) and may also account for their central depressant properties (Seeman, 1972). In spite of this, there is no systematic information on the partitioning of barbiturates into membranes of defined composition, and most studies requiring such information have relied upon partition coefficients from simple solvents. The reliability of the latter manoeuvre is questionable, however, for depending on the choice of solvent, the absolute value of the partition coefficient may vary by orders of magnitude, and even the ranking order of a number of barbiturates in different solvents may change (Kakemi, Arita, Hori & Konishi, 1967). In the face of these uncertainties a systematic study of the dependence of the partition coefficients of barbiturates on the lipid composition of membranes seems long overdue and ought to provide a firmer foundation for the assessment of both their pharmacodynamics and the specificity of their action on cellular structures.

In this paper we report the membrane-buffer partition coefficients of 5-ethyl-5-(1-methylbutyl) barbituric acid (pentobarbitone) in lipid bilayer membranes of various compositions. Lipid bilayer membranes were chosen, rather than a series of biomembranes, both because their lipid composition can be systematically varied and because they avoid the problem of simultaneous lipid and protein interactions with the drug. Since barbiturates are weak acids with pKs in the physiological range, their pharmacodynamics (Brodie *et al.*, 1960) and anaesthetic action (Narahashi, Frazier, Deguichi, Cleaves & Ernam, 1971) are pH-dependent, and we have therefore also examined the influence of pH on the membrane-buffer partition coefficients.

Methods

Two forms of lipid bilayers could be used for these studies. First, strong agitation or brief sonication of an aqueous lipid dispersion results in the formation of a large number of concentric spherical lipid bilayer shells separated by aqueous compartments. Second, upon more prolonged sonication these multi-lamellar vesicles form monolamellar vesicles with single internal aqueous compartments (Bangham, 1972). Both types of vesicles have been used in solubility studies. The monolamellar vesicles have the advantages of being of well defined size and structure and of requiring that the agent in question only penetrate one phospholipid bilayer to achieve complete equilibration with the lipid and internal compartment. On the other hand, these vesicles are not readily sedimented in the centrifuge and consequently a dialysis bag has been employed in the past in order to determine the buffer concentration of the agent in equilibrium with the membrane suspension. This increases the equilibration time and reduces the sensitivity of the method, and in consequence use of monolamellar vesicles in partitioning studies has been restricted to agents of high partition coefficients such as steroids (Heap, Symons & Watkins, 1970). The readily centrifuged multilamellar vesicles have thus been used in most studies previously reported. However, we found them to impose unacceptably long equilibration times, particularly in view of the possibility of lipid oxidation. To overcome these shortcomings we describe the use of an ultrafiltration method which allows partition coefficients to be studied in monolamellar vesicles with a sensitivity comparable to that generally obtained with multilamellar vesicles.

Materials

Phospholipids from egg yolk were obtained from Lipid Products, U.K., and were tested for purity by thin layer chromatography. Cholesterol was Sigma's chromatographic grade and was recrystallized in methanol twice before use to yield a m.p. of 148–9°C *in vacuo*. Radioisotopes were from New England Nuclear.

Procedure

About 5 μmol of the appropriate lipids for each ml of final suspension were mixed in organic solvents and evaporated to dryness. They were resuspended in 0.15 M KCl appropriately buffered (e.g. 10 mM Tris-HCl) and sonicated to clearness in a graduated centrifuge tube under nitrogen at 20°C in a sonicating bath (Heat systems, Model 5 x 5, 40 kHz). Aliquots of this suspension were added to buffer solution containing 0.2–0.8 mM pentobarbitone including 0.1 μCi of [^{14}C]-pentobarbitone. The pH was checked before and after incubation; adjustment of the pH was particularly necessary with suspensions containing phosphatidic acid. Equilibration, which was actually achieved within an hour or so, was normally allowed to proceed overnight in magnetically stirred bottles which were sealed under nitrogen and placed in a water bath maintained at 25.0 ± 0.05°C. The oxidation index (Klein, 1970) of the phospholipids did not increase during this procedure. An aliquot of the equilibrated suspension was taken for phosphate determination (McClare, 1971). A second aliquot was filtered through an ultrafilter (Amicon, XM-50) in a 10 ml filtration cell (Amicon model 12) thermostated in the water bath, the first 0.5 ml of filtrate being discarded and the remainder analysed by liquid scintillation counting in a Nuclear Chicago, Mark II system. Control solutions, not equilibrated with lipid vesicles, were treated in the same way to yield the initial or total pentobarbitone concentration. Corrections for adsorption on the filter were less than 2% of the final partition coefficient. Two controls were performed in early experiments to ensure that the filter was achieving complete separation. First, no phosphate could be detected in the filtrate and second, when [^{14}C]-cholesterol was incorporated in the vesicles in the absence of [^{14}C]-pentobarbitone, no radioactivity above background was detected in the filtrate.

A few experiments were carried out at elevated pressure. Equilibration was carried out in small stainless steel bombs. Since filtration at high pressure was not possible, 0.5 ml of liposome suspensions in dialysis bags were equilibrated in 10–15 ml of buffer containing pentobarbitone in glass tubes sealed with a serum cap and stirred magnetically. One atmosphere controls were treated similarly in an unpressurized bomb. Hydrostatic pressure was raised by hand pump and measured on a bourdon tube gauge to ± 5%. Equilibration was for 18 hours. Depressurization, opening the bombs and removal of the dialysis bags was achieved always within 1 to 5 minutes. The rate of equilibration of pentobarbitone through the dialysis bag was too slow to introduce errors during this manoeuvre.

The partition coefficient was calculated from the difference between the initial and final buffer concentration and the concentration of lipid present in the suspension. The coefficient is expressed as the ratio (mol of pentobarbitone per g of lipid/mol of pentobarbitone per ml of buffer). The molecular weight of egg phosphatidylcholine was taken as 780 daltons. However, in Figures 1 and 2 only the partition coefficient is given per g of phospholipid (omitting any incorporated cholesterol), this being the direct experimental variable.

Results

A comparison between the partition coefficient obtained with the ultrafilter and that obtained with the conventional centrifugation method was performed using multilamellar vesicles of phosphatidylcholine at pH 7.9 in both cases; these methods yielded values of 98 and 100, respectively, which are not detectably different. Comparison of single shelled phosphatidylcholine vesicles at pH 7.9, separated from the buffer either by filtration or by a dialysis bag, yielded values of 104 and 105 respectively.

The measured membrane/buffer partition coefficient is almost independent of the pentobarbitone concentration in the buffer over the range 0.15–2.0 mM (Figure 1). The small, but consistent, decrease observed is statistically significant ($P<0.002$) only when the data are pooled. The data show that the deviations from the ideal are very slight in this concentration range where the mol fractions of pentobarbitone dissolved in the lipid range from about 0.2 for phosphatidylcholine to 0.02 for phosphatidylcholine : cholesterol 1:1.

The partition coefficient (λ) of the acid and its anion may be derived by measuring the apparent λ over a range of pH. In Figure 2 the results of such studies are presented for two membranes. The relation between the apparent λ and the degree of dissociation is linear within experimental error, suggesting that the acid and its anion interact independently with the membrane. The partition coefficient for the acid, λ^{HA}, and the anion λ^{A^-}, derived from the intercepts of such plots are given in subsequent figures. The magnitude of the partition coefficient for the anion was not entirely expected and was accordingly checked in experiments with multilamellar vesicles, which were separated from buffer after equilibration by either centrifugation or ultrafiltration. In addition to scintillation counting a spectroscopic assay of the barbiturate was also employed (Williams & Zak, 1959). These experiments gave identical results with our usual method, yielding for phosphatidylcholine vesicles a λ^{A^-} of 9 ± 2.5. Our value of λ^{A^-} is thus not an artifact of the filtration procedure, nor attributable to possible chemical degradation products of the labelled barbiturate.

The effect of varying the lipid composition of the bilayer membrane was next examined. Cholesterol was incorporated into phosphatidylcholine bilayers in amounts up to the maximum obtainable (50 mol %) and caused the partition coefficient of the undissociated molecule to fall, sharply at first, and then more slowly, whilst that of the anion was unaffected (Figure 3). Incorporation of up to 38 mol % of the negatively charged lipid, phosphatidic acid, into phosphatidylcholine bilayers also caused a reduction in the partition coefficient of the undissociated pentobarbitone.

The total ionic strength of the buffer solution was varied by changing the KCl concentration from 0 to 0.31 M. At high pH the partition coefficient in

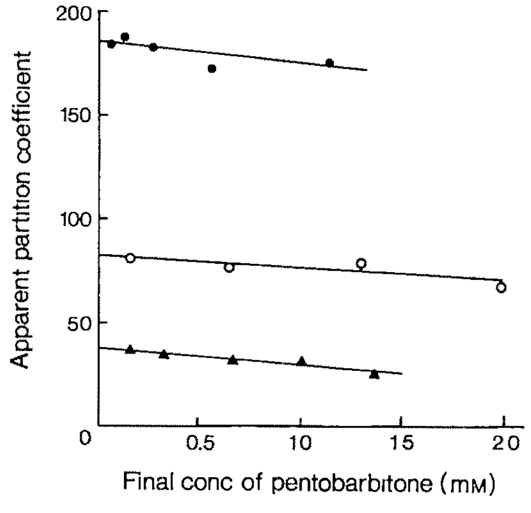

Figure 1 The dependence of the membrane/buffer partition coefficient at pH 6.95 upon the concentration of free pentobarbitone in the buffer. The partition coefficients in this figure and Figure 2 are expressed per g of phospholipid, not total lipid. (●) Phosphatidylcholine; (○) 72% phosphatidylcholine, 28% cholesterol; (△) 50% phosphatidylcholine, 50% cholesterol. Proportions of lipids are expressed on a molar basis.

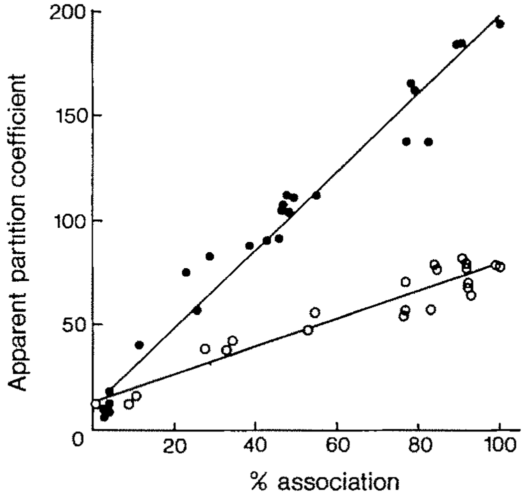

Figure 2 The dependence of the membrane/buffer partition coefficient (per g of phospholipid) on the degree of association of pentobarbituric acid (pKa=8.0). (●) Phosphatidylcholine; (○) 75% phosphatidylcholine, 25% cholesterol.

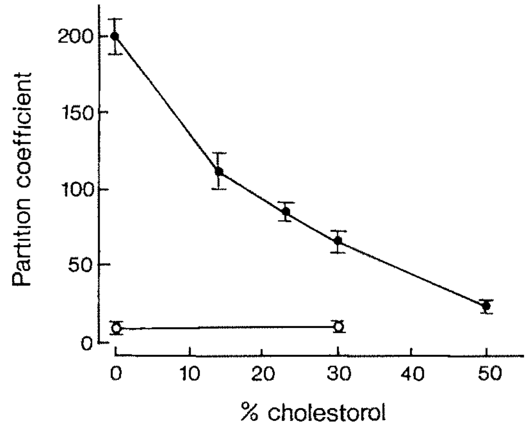

Figure 3 The dependence of the partition coefficient of pentobarbitone on the cholesterol content of phosphatidylcholine bilayer membranes. Partition coefficients are per g of total lipid (phosphatidylcholine + cholesterol). (●) Low pH; (O) high pH.

phosphatidylcholine bilayers was not markedly dependent on ionic strength, but the partition coefficient of the anion is apparently reduced to zero at high ionic strength. In contrast, at low pH a small decrease was observed at low ionic strength in phosphatidic acid-containing membranes (Figure 5).

Experiments with dialysis bags in the pressure bombs gave identical results to those obtained normally, but the standard deviations were much higher (between 10 and 20% of the mean). In three experiments at pH 7.8 the ratio of partition coefficient at one atmosphere to that at 400 atm was 1.13 for phosphatidylcholine and 0.99 for phosphatidylcholine containing 30% cholesterol. No significant effect of pressure was observed therefore.

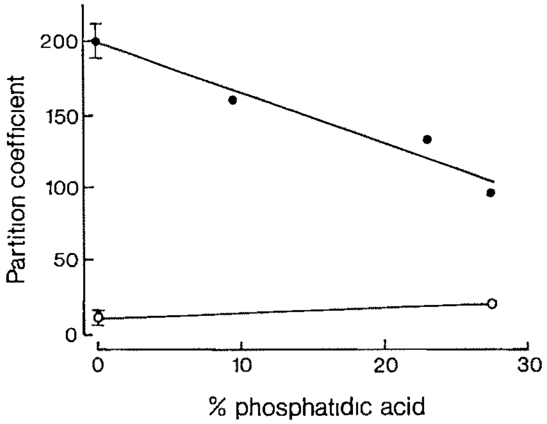

Figure 4 The dependence of the partition coefficient of pentobarbitone on the phosphatidic acid content of phosphatidylcholine bilayer membranes. (●) Low pH; (O) high pH.

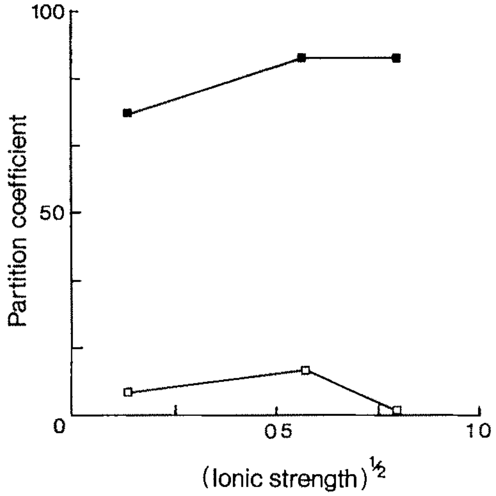

Figure 5 The effect of ionic strength. The relation between partition coefficient and the square route of ionic strength for (■) 30% phosphatidic acid, 70% phosphatidylcholine membranes at pH 4 0; (□) phosphatidylcholine membranes at pH 9.6.

Discussion

The filtration technique gave values comparable to those we obtained by the conventional centrifugation technique. The main advantages of the method are the ease of equilibration with the monolamellar compared to the multilamellar vesicles, particularly with charged solutes, and the closer control of temperature possible during filtration compared to centrifugation. One drawback is the tendency for lipid, when present in high concentrations, to clog the filter. We also found that the combination of charged lipids and charged solutes can cause problems, particularly at low ionic strength, which probably result from an interaction between absorbed lipid and solute anion during passage through the filter.

One interesting observation from the control experiments was that the multilamellar vesicles consistently gave slightly lower partition coefficients than monolamellar ones. This was not an experimental artifact arising from the greater difficulty of obtaining equilibration in the multilamellar vesicles because the effect was observed with both the uncharged and the charged solutes (which are presumably less permeable and thus slower to equilibrate). A possible explanation is that the more fluid structure of the highly curved monolamellar vesicles (diameters in the range of

20–55 nanometres: Johnson, 1973) provides a better solvent for the barbiturate because less work has to be done to introduce the barbiturate between the lipid molecules.

The finding that the partition coefficient is independent of pentobarbitone concentration (Figure 1) is consistent with that obtained by Roth & Seeman (1972) in erythrocyte ghosts. This implies either that there are no free discrete binding sites at these concentrations (i.e. the membrane behaves as a homogeneous solvent), or that there are a very large number of such sites so that only a small fraction are saturated in the concentration range studied. The limited solubility of pentobarbitone prevents studies at significantly higher concentrations. The conclusions apply essentially to the uncharged solute since the data in Figure 1 are at pH 6.95 (pK of pentobarbitone is 8.0). Because the partition coefficient of the anion was not large compared to our errors it was not studied over a wide concentration range.

The effect of ionic strength at low pH (Figure 5) was similar in form to that reported for erythrocyte ghosts (Roth & Seeman, 1972). The binding of the anion is almost completely displaced by high ionic strength suggesting that surface electrostatic forces, perhaps between the anion and the charged choline group of the lipid (Blaustein & Goldman, 1966), predominate and that the anion does not significantly penetrate the bilayer. The A^-:phosphatidylcholine ratio is about 1:100 at 1 mM barbiturate and 0.16 M salt. At higher concentrations, charge-charge repulsion would limit the binding.

The effects of cholesterol and phosphatidic acid on the partition coefficient are illustrated in Figures 3 and 4 respectively. The marked decrease in partition coefficient for the uncharged barbiturate with increasing cholesterol content is similar to, but more pronounced than, that reported for benzyl alcohol (Colley & Metcalfe, 1972) and suggests that this may be a general phenomenon for small molecules. This is supported by the observation that incorporation of cholesterol into phospholipid bilayers reduced their permeability to small molecules (de Geir, Mandersloot & van Deenan, 1969).

There were no gross effects of cholesterol on the partition coefficient of the anion, though small trends would not be apparent with the level of precision obtained in these measurements because the observed coefficient was so small.

The effect of incorporating a negatively charged lipid, phosphatidic acid, which has been prepared from phosphatidylcholine from the same source as that used here and thus has the same acyl composition, is roughly parallel to that of cholesterol. Such an effect has apparently not been described previously.

The origin of the effects of cholesterol and phosphatidic acid on the phosphatidylcholine-buffer partition coefficient cannot be directly deduced from work of this nature. That would require a detailed spectroscopic study. However, it seems probable that a molecule with the amphiphilic characteristics of a barbiturate would locate primarily with the pyrimidine ring in the polar interfacial region of the membrane and the 5-alkyl substituents in the apolar interior, whilst a smaller proportion of molecules might be enclosed entirely within the apolar region. Nuclear magnetic resonance studies have been interpreted to support such a model for benzyl alcohol (Colley & Metcalfe, 1972).

The anaesthetic effect of pentobarbitone is reversible by pressure (Miller, 1972). Within the framework of the membrane hypothesis of anaesthetic action, this might arise because pressure displaced the anaesthetic from its site of action, or because pressure opposed the anaesthetic-induced membrane expansion. Mullins (1954) showed that the former possibility could not be ruled out, although it subsequently has been for gaseous anaesthetics (Miller, Paton, Smith & Smith, 1973). Our demonstration that high pressures do not appreciably alter pentobarbitone's partition coefficient is not consistent with the displacement model. Since the variation of partition coefficient with pressure reflects the difference between the partial molar volume of pentobarbitone in lipid and buffer, we may also conclude that this difference is small.

Our findings, if they can be extrapolated to biomembranes, raise an interesting question of specificity for the lipid theory of anaesthetic action. Neuronal membranes contain high proportions of cholesterol and charged lipids (Whittaker, 1969) and thus will tend to harbour lower concentrations of anaesthetic than other membranes such as mitochondria. In the case of pentobarbitone the observation that only membranes relatively high in cholesterol are fluidized may go some way towards answering this question, but for other anaesthetics this explanation is not possible (Miller & Pang, 1976).

How far may the lipid bilayer partition coefficients measured here be used to predict the values for biological membranes? The lipids of erythrocyte ghosts are 45 mol % cholesterol, and 17% of the phospholipids are negatively charged. The neutral phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in roughly equal amounts. Phosphatidylserine is the major negatively charged lipid (Guidotti, 1972). If we segregate the lipids into three classes, neutral phospholipids, negatively charged phospholipids and cholesterol, and make the assumptions that these classes may be represented by phosphatidylcholine, phosphatidic acid and cholesterol, and, further, that the effects of these lipids are additive, we may use our lipid bilayer data to make a prediction of the partition coefficient in the erythrocyte ghost. Thus from Figure 4, λ is 138 when the negative lipid is 17%. Figure 3

Table 1 A comparison of partition coefficients measured in lipid bilayers, erythrocytes and bulk solvents

Partition coefficient in:	*Pentobarbitone*	*Benzyl alcohol*
Phosphatidylcholine bilayers	200 a	18 b
Phosphatidylcholine : cholesterol (1:1) bilayers	21 a	7 b
Erythrocytes	11 c,d	4 c
Octanol	89 e	13 e
Carbon tetrachloride	0.9 f	—

(a) This work, (b) Colley & Metcalfe, 1972; (c) Seeman, 1972; (d) Korten & Miller, 1976; (e) Hanch & Anderson, 1967; (f) Kakemi *et al.*, 1967.

shows the ratio of λ for phosphatidylcholine to that for 45% cholesterol in phosphatidylcholine is 0.17, which, under the above assumptions, yields an estimated λ for the phosphatidylcholine : cholesterol : phosphatidic acid bilayer of 23. The partition coefficient of red blood cell ghosts has been measured at low pH; values are 9.6 (Roth & Seeman, 1972) and 12 (Korten & Miller, 1976). Since only 44% by weight of the erythrocyte ghost membrane is lipid, and we further assume that negligible amounts of pentobarbitone are bound to the protein, then these values become 22 and 27 for the partition coefficient in the lipid bilayer regions of the erythrocyte ghost membrane. Such good agreement with our estimate based on the lipid bilayer is probably partly fortuitous, because the assumptions we have made are extremely crude; for example, the lipids in the red blood cell are not homogeneously distributed (Bretcher, 1973; Gordesky & Marinetti, 1973). Nevertheless the little systematic data available (Table 1) suggests that the lipid bilayer approach offers much better estimates of the absolute value of membrane partition coefficients than an approach based on model solvents. We propose that knowledge of a few readily measured lipid bilayer partition coefficients for a given solute should provide a relatively reliable means for predicting its partition coefficient in any biomembrane, or region of a biomembrane, whose composition is known. This procedure is more rational than that based on the use of model solvents, such as octanol (Leo, Hanch & Elkins, 1971), and deserves further investigation.

This work was supported by grants from the National Institutes of Neurological Diseases and Stroke and of General Medicine of the U.S.A.

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(Received November 5, 1976.
Revised February 10, 1977.)

Br. J. Pharmac. (1977), **61**, 65–76

SUPPRESSION OF TRANSMISSION OF NOCICEPTIVE IMPULSES BY MORPHINE: SELECTIVE EFFECTS OF MORPHINE ADMINISTERED IN THE REGION OF THE SUBSTANTIA GELATINOSA

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1 In spinal cats anaesthetized with α-chloralose, a study was made of the effects of morphine and naloxone, administered electrophoretically from micropipettes, on the responses of dorsal horn neurones to noxious (raising of skin temperature above 45°C) and innocuous (deflection of hairs) peripheral stimuli.

2 Administered near cell bodies, morphine reduced the nociceptive responses of only 2 of 37 cells. Excitation occurred more commonly than depression and abnormalities in action potentials were commonly observed following ejection of morphine. None of these effects of morphine was antagonized by electrophoretically applied naloxone.

3 Administered in the substantia gelatinosa from one micropipette while recording responses of deeper neurones with a second micropipette, morphine reduced the nociceptive responses of 15 of 19 neurones. Firing in response to deflection of hairs was not reduced by morphine. Depression of nociceptive responses by morphine was long lasting (>20 minutes). Naloxone ejected into the substantia gelatinosa or given intravenously in doses as low as 0.1 mg/kg antagonized the effects of morphine. The effectiveness of this dose of intravenous naloxone suggests that the concentrations of morphine in the substantia gelatinosa which reduced nociceptive responses were not unlike those present after analgesic doses of systemic morphine. Naloxone alone, and excitant and depressant amino acids ejected into the substantia gelatinosa had little effect on cell firing.

4 Both the selective action of morphine on nociceptive responses and the reversal of this action by intravenous naloxone suggest that the opiate receptor present in the substantia gelatinosa is relevant to analgesia produced by opiates given systemically.

Introduction

When administered intravenously, morphine impairs spinal transmission of impulses in small, but not large, diameter primary afferents (Wikler, 1945; Koll, Haase, Block & Mühlberg, 1963; McLane & Martin, 1967b; Besson, Wyon-Maillard, Benoist, Conseiller & Hamann, 1973; Le Bars, Menétrey, Conseiller & Besson, 1975; Jurna & Grossman, 1976; Le Bars, Guilbaud, Jurna & Besson, 1976). Since nociceptive fibres are of small diameter (Iggo, 1962; Bessou & Perl, 1969) this spinal action may be a significant component of analgesia produced by morphine, particularly as in man this alkaloid has minimal effects on other sensations such as touch, vibration, vision, hearing and olfaction (Wikler, 1950; Kreuger, 1955). The recent finding that, in rats, restricted superfusion of the spinal cord with morphine produces analgesia localized to the appropriate skin segments supports this suggestion (Yaksh & Rudy, 1976).

In attempting to explain the effects of morphine on the spinal cord, several investigators have ejected morphine and the narcotic antagonist naloxone electrophoretically from micropipettes in the vicinity of spinal neurones. The results have not been uniform. Calvillo, Henry & Neuman (1974) found that, in cats anaesthetized with α-chloralose, morphine depressed the firing of neurones excited by noxious heat but not that of cells unresponsive to this stimulus. Antagonism of this effect by electrophoretic naloxone was, however, observed with only 2 of 7 cells. As the excitation of 'nociceptive' neurones by L-glutamate was also reduced, morphine probably had a postsynaptic depressant effect. In contrast, Dostrovsky & Pomeranz (1973) found that morphine depressed excitation by L-glutamate and L-aspartate, but not the spontaneous and synaptically induced firing of dorsal horn neurones of spinal cats. No distinction was made between cells on the basis of response to noxious stimuli, and these authors have subsequently reported that naloxone does not antagonize this depressant action of morphine (Dostrovsky & Pomeranz, 1976).

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Zieglgänsberger & Bayerl (1976) also found morphine to depress excitation of dorsal horn neurones by L-glutamate, but, in addition, spontaneous firing and responses to tactile stimulation were reduced. Prior ejection of naloxone blocked these actions of morphine. Noxious stimuli were not used in these experiments, and the animals were either decerebrate or anaesthetized with sodium pentobarbitone. Depression of dorsal horn neurones by morphine was not blocked by naloxone in the experiments of Duggan, Davies & Hall (1976) using cats anaesthetized with pentobarbitone. In addition dextrorphan, an inactive isomer of the opiate levorphanol, was a more potent depressant of cell firing than either morphine or levorphanol.

Most of the experiments quoted employed multibarrel micropipettes in which the orifices of recording and drug ejecting pipettes were at the same level. Drug administration would thus be predominantly in the region of cell bodies. In the micropipette assemblies of Zieglgänsberger & Bayerl (1976) the recording pipette projected 10–20 µm beyond the drug administering pipette and hence receptor sites other than those on cell bodies may have been reached.

In the present experiments, morphine and naloxone were administered electrophoretically in two ways. Firstly, from conventional multibarrel micropipettes, and thus in the vicinity of cell bodies of dorsal horn neurones activated by both noxious and non-noxious stimuli to the skin. When it became apparent that the results from this method of administration were not relevant to the effects of systemic opiates it was decided to administer both morphine and naloxone to the dendritic regions of neurones of spinal laminae IV and V. The dendrites of cells of these laminae project into the substantia gelatinosa where contacts are made with primary afferent fibres, descending fibres and axons of the intrinsic neurones of this area (Cajal, 1909; Szentágothai, 1964). Axo-axonic contacts are abundant in the substantia gelatinosa and such contacts are an important element of the 'gate control' theory of pain of Melzack & Wall (1965). The results from these experiments can be related to analgesia produced by morphine. Preliminary observations have been published (Duggan, Hall & Headley, 1976).

Methods

Experiments were performed on 36 cats (2.5–3.5 kg); 32 were anaesthetized with α-chloralose, 50 mg/kg intraperitoneally, 3 were decerebrated by midbrain coagulation under halothane anaesthesia, and the remaining animal received initially an intraperitoneal injection of sodium pentobarbitone, 35 mg/kg.

The spinal cord was exposed by lumbar laminectomy and in all experiments was sectioned at the first lumbar segment. Blood pressure was monitored continuously and experiments were terminated if mean systolic pressure fell below 100 mmHg. Animals were paralysed with gallamine triethiodide and were artificially ventilated with air, end tidal CO_2 levels being maintained at approximately 4%.

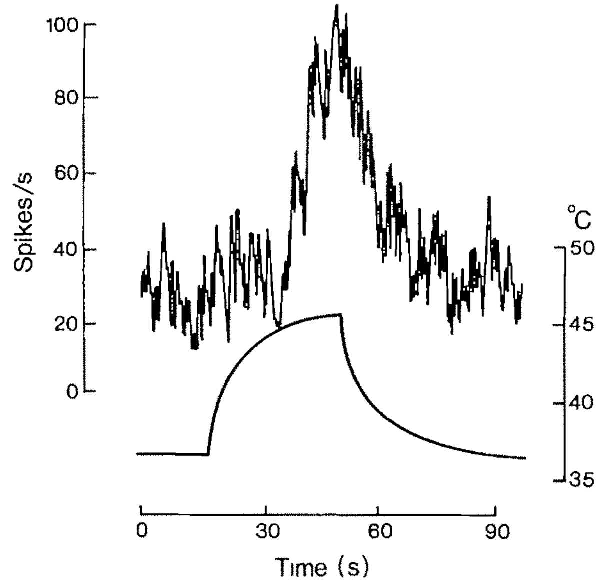

Figure 1 Firing of a lamina IV spinal neurone in response to radiant heat to the fourth left digital foot pad in the cat. Upper record; cell firing measured with a ratemeter. Lower record; skin surface temperature measured with a thermistor placed at the edge of the heated area.

These experiments sought to study quantitatively the effect of morphine on the firing of dorsal horn spinal neurones by impulses in nociceptive and non-nociceptive afferents.

Nociceptive afferents were activated by radiant heat to the left hind limb. There is evidence (Beck, Handwerker & Zimmermann, 1974) that when skin temperature is raised above 45°C the input to the spinal cord from the heated area is almost exclusively from thermal nociceptors. The posterior surface of the left hind limb was shaved, blackened with ink and held in an extended position. Radiant heat from a 45 W lamp was focused with a condenser lens to an area of approximately 3 mm diameter. Skin surface temperature was monitored by a thermistor placed at the edge of the heated area and was displayed on a pen recorder together with cell firing produced by this stimulus. Figure 1 illustrates the relationship between skin surface temperature, measured in this way, and cell firing for neurones included in Results. The firing of this neurone was not increased above the spontaneous level until a skin temperature of 45°C was reached and outlasted the stimulus by approximately 20 seconds. Cells which fired with a latency shorter than that illustrated and those in which firing declined with skin temperatures above 45°C were excluded from analysis. Repeated nociceptive stimuli of this type have been reported to result in increased firing of nociceptors (Perl, Kumazawa, Lynn & Kenins, 1976). Enhanced activation of neurones was uncommon when heat stimuli of 25–30 s were repeated at intervals of 2 minutes.

Innocuous stimulation of skin was produced either by light touch to the shaved and unshaved areas using a camel hair brush, or, when quantitative responses were required, by deflection of hairs by an air jet moved by the armature of a relay activated by a Grass S8 stimulator (pulse frequency 2–4 Hz, mark: space ratio 1:1). The firing produced by these stimuli consisted of a group of action potentials commonly followed by a period of inhibition of spontaneous firing. When used for quantitative studies, the frequency of movement of the air jet was selected not only on the basis of the rapid adaptation of the peripheral receptors (Brown & Iggo, 1967) but also so that the number of evoked action potentials was not diminished by the inhibition following the preceding deflection. The time constant of the recording system (0.5 s) effectively integrated these responses (Figures 3a, 4, 6).

An automatic timer controlled both the filament current to the lamp producing noxious stimulation and the air supply to the relay device. Most results were obtained from cells activated by heat applied to a digital pad and by an air jet directed at adjacent interdigital hairs.

In the first series of experiments extracellular recordings of the firing of dorsal horn neurones were obtained with the 4 M NaCl filled centre barrels of seven barrel micropipettes. The outer barrels contained different combinations of the following compounds to be ejected electrophoretically: L-glutamate Na (0.5 M, pH 7); L-aspartate Na (0.5 M, pH 7.5); DL-homocysteate Na (0.2 M, pH 7); glycine (0.5 M, pH 3); γ-aminobutyric acid (GABA, 0.5 M, pH 3); morphine sulphate (0.07 M); naloxone hydrochloride (0.1 M in water or 0.1 M NaCl); pontamine sky blue (2% in 0.5 M Na acetate). Drugs were thus administered in the region of cell bodies.

In the later experiments, drugs were ejected in the region of the substantia gelatinosa from a seven barrel micropipette assembly, the tip of which was separated from that of a five barrel micropipette, used to record activity of deeper neurones, by a known distance.

The outer barrels of the seven barrel micropipettes contained combinations of the following solutions: morphine sulphate (0.07 M), naloxone hydrochloride (0.1 M), glycine (0.5 M, pH 3), γ-aminobutyric acid (GABA, 0.5 M, pH 3), DL-homocysteate Na (0.2 M, pH 7), L-glutamate Na (0.5 M, pH 7), pontamine sky blue (2% in 0.5 M Na acetate), acid fast green (saturated solution in 2 M NaCl).

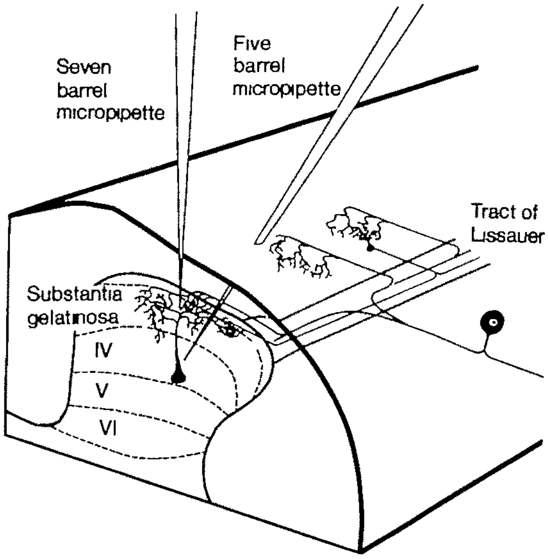

Figure 2 Arrangement of micropipettes for administering drugs in the substantia gelatinosa of the cat while recording the activity of deeper spinal neurones. The dendrites of a lamina IV neurone are shown establishing contacts with terminals of a primary afferent fibre and terminals of a substantia gelatinosa neurone of another segment. The passage of these fibres in the tract of Lissauer is also shown. The angle between the 5 and 7 barrel micropipettes was 18°.

The outer barrels of the five barrel micropipettes contained combinations of DL-homocysteate Na (0.2 M, pH 7), L-glutamate Na (0.5 M, pH 7), glycine (0.5 M, pH 3), and acid fast green (saturated solution in 2 M NaCl). The centre barrels of both types of micropipette were filled with 4 M NaCl for recording purposes.

The procedure used for positioning the two micropipettes independently was as follows. By the use of a stereoscopic microscope, the two micropipettes were first aligned tip to tip, 50–200 μm above the surface of the cord, and the appropriate micromanipulator readings were noted. The seven barrel pipette, with which extracellular activity was also recorded, was then lowered vertically as far as spinal lamina I. Ejection of dye showed that this lamina could be reliably identified by the appearance of cells responding to electrophoretic DL-homocysteate or L-glutamate and to cutaneous stimulation. The electrode was then advanced a further 50–200 μm. Drugs were thus ejected in spinal laminae II and III, the laminae usually accepted as comprising the substantia gelatinosa (Szentágothai, 1964; Scheibel & Scheibel, 1968). The distance of this final site from the convergence point above the cord was then measured. The second pipette, of 5 barrels, angled 18° caudally, was then inserted in the spinal cord at an appropriate distance rostrally so that it would pass a defined distance vertically below the tip of the substantia gelatinosa pipette. The planned vertical separation of tips ranged from 150 to 400 µm (300 µm was most commonly used) but, because it was exceptional to locate a neurone precisely when this planned separation had been reached, it was decided to study neurones located approximately 100 µm before and up to 500 µm beyond this point. Actual tip separations varied from 150–680 µm with two thirds lying in the 300–500 µm range.

The relationship of the two micropipettes is shown diagramatically in Figure 2.

The relative positions of the two electrode tips were verified for one pair of positions in each experiment by deposition of dye, either acid fast green or pontamine sky blue. Dyespots were subsequently located in 50 µm frozen sections.

Neuronal firing rates, measured with a ratemeter, were displayed continuously on a pen recorder. In later experiments the total number of action potentials evoked by noxious heat and hair deflection was determined with a gated electronic counter. With nociceptive responses the gate duration needed to outlast the stimulus by several seconds whereas with hair deflection the gate and the stimulus were usually of the same duration. Integrals determined in this way are affected by changes in spontaneous firing and since the gate durations were different for the nociceptive and non-nociceptive responses the two will be unequally affected. This was allowed for by subtracting from each response a number determined by the gate duration and the mean spontaneous firing just prior to each response (see Figure 6). In earlier experiments where response integrals were not determined response amplitude, in spikes per s, was plotted with respect to time.

Results

A. Morphine and naloxone administered in the region of cell bodies

An attempt was made to sample neurones in several areas of the dorsal horn. Table 1 summarizes the responses of neurones of the various layers of Rexed to noxious and innocuous skin stimulation.

In agreement with others (Handwerker, Iggo & Zimmermann, 1975; Price & Browe, 1975) the majority of cells were excited by both types of stimulus and hence the terms 'nociceptive' and 'non-nociceptive' have not been used to categorize neurones.

(i) Effects of electrophoretic morphine.

(a) Spontaneous firing. Ejected with currents of up to 80 nA, morphine had no effect on the spontaneous firing of the five neurones of lamina I, excited 2 of 14 neurones of lamina IV and excited 5 of 13 neurones of lamina V. Inhibition of spontaneous firing was not seen with cells of these superficial laminae. In laminae VI and VII, 4 neurones were inhibited, 4 excited and 5 unaffected. No correlation was found between the responses of these neurones to skin stimulation and the effect of morphine on spontaneous firing.

During these experiments, the ejecting current of morphine was increased in a stepwise manner till abnormalities in action potential amplitude and configuration appeared (Duggan & Curtis, 1972). This occurred with currents of the order of 60 to 80 nA. Excitation by morphine, which occurred with the higher ejecting currents, took the form of firing in groups of action potentials (bursts) and thus differed from that previously observed with Renshaw cells (Davies & Duggan, 1974).

(b) Firing by noxious and innocuous skin stimulation. Morphine, ejected with increasing currents of up to 70 nA, depressed the nociceptive responses of only 2 of 37 cells tested. With 9 cells the response was enhanced together with an increase in spontaneous firing. To test for possible selectivity in the effects of morphine towards nociceptive responses, 6 cells were activated alternately by noxious heat to an area of the left hind limb and by a moving air jet directed at adjacent hairs. In no case was the response to either sensory modality significantly reduced during morphine ejection. With 3 neurones responses to both

Table 1 Responses to skin stimulation of dorsal horn neurones classified by laminae of Rexed

		Responses to skin stimulus					
		Excited by noxious heat only	*Excited by innocuous mechanical only*	*Excited by noxious and innocuous*	*Inhibited by noxious heat*	*Not affected*	*Totals*
I	I			5			5
Rexed	IV		1	11	1	1	14
Lamina	V			12		1	13
	VI & VII	1	2	4	4	2	13
Totals		1	3	32	5	4	45

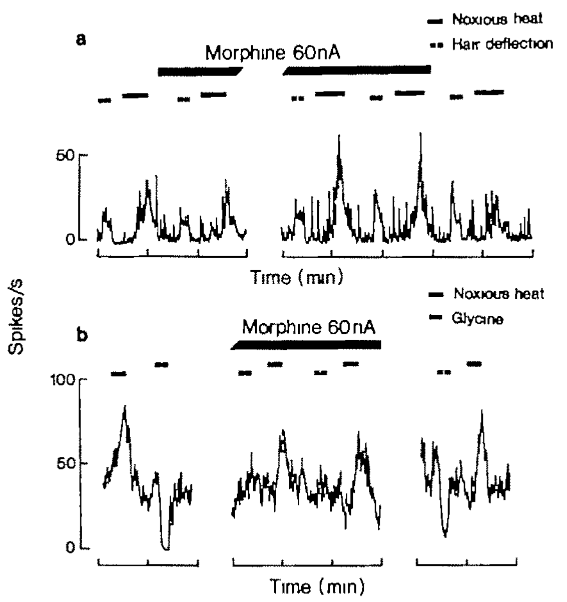

Figure 3 (a) Effect of electrophoretic morphine on the excitation of a lamina V spinal neurone of the cat by noxious heat to the fourth digital pad of the left hind limb and by deflection of adjacent hairs by a moving air jet. Morphine was ejected for a total of 7 minutes. (b) Failure of concentrations of morphine, adequate to antagonize depression of cell firing by glycine, to reduce activation of a lamina VI spinal neurone by noxious heat. The three sets of records are, from left to right, before, during and 2 min after ejection of morphine. Noxious heat was applied to the fourth digital pad of the left hind limb. Morphine was ejected for 8 min before the period illustrated.

stimuli were enhanced and this was associated with irregular firing in bursts (Figure 3a).

Morphine blocks the inhibitory action of glycine on spinal neurones (Curtis & Duggan, 1969). As a further safeguard that the essentially negative results presented above were not due to failure to eject an adequate amount of morphine, on five cells depression of firing by glycine was alternated with excitation by noxious heat. In all cases currents of morphine more than twice those adequate to block the action of glycine had no significant effect on activation by noxious heat (Fig. 3b).

(c) Excitation by L-glutamate. The effects of morphine on excitation by L-glutamate were variable. Of 13 cells excited both by noxious and innocuous stimulation of skin, morphine (ejecting currents of up to 60 nA), enhanced the response to glutamate of 5 cells and this was accompanied by increased spontaneous firing. A further increase in excitation by L-glutamate occurred immediately after the termination of morphine ejection. Morphine depressed the excitation by L-glutamate of 7 cells but this was associated with abnormalities in action potential amplitude and configuration.

(ii) Effects of electrophoretic naloxone. Ejected with increasing currents of up to 60 nA, naloxone had no significant effect on spontaneous firing and responses to skin stimulation. When morphine had an effect on cell firing, either excitation or depression, no antagonism by naloxone was observed (10 neurones). Attention needs, however, to be drawn to an apparent antagonism of morphine which occurred in these tests.

Naloxone reduced the excitation by L-glutamate of 4 of 7 cells, but, following the termination of ejection, with 5 neurones the responses to L-glutamate were enhanced by up to 100% above control values for as long as 10 minutes. When responses to L-glutamate had been depressed by morphine, simultaneously administered naloxone either had no effect or further reduced the action of the amino acid. When the ejection of naloxone was terminated, however, (that of morphine continuing) excitation by L-glutamate was greater than that prior to ejection of naloxone. Such an action cannot be interpreted in terms of antagonism between morphine and naloxone as it occurred in the absence of morphine.

B. Morphine and naloxone ejected in the region of the substantia gelatinosa

(i) Distribution of cells studied. Cells were chosen on the basis of distance from the drug-administering micropipette and reproducibility of responses to both nociceptive and non-nociceptive stimuli. Twenty one were in Rexed lamina IV, and nine in lamina V.

(ii) Morphine and responses to noxious heat and deflection of hairs. Morphine, ejected in the region of the substantia gelatinosa with currents of 30–250 nA, reduced the response to noxious heat of 12 of 15 cells activated alternately by deflection of hairs and noxious heat to the appropriate part of the hind limb. With 3 neurones neither response was reduced. Of the 12 neurones with which nociceptive activation was reduced by morphine, the non-nociceptive response was unchanged with 7 cells and increased above controls with 5. With a further 4 cells nociceptive responses alone were studied and morphine reduced these in 3.

Unlike the experiments on cell bodies, where ejecting currents of morphine of the order of 60–80 nA caused abnormalities in spike configuration, when morphine was administered in the substantia gelatinosa, the relatively large currents (mean 146 nA ± 62 nA s.d.) needed to reduce nociceptive responses did not affect action potentials.

Depression of nociceptive activation by morphine

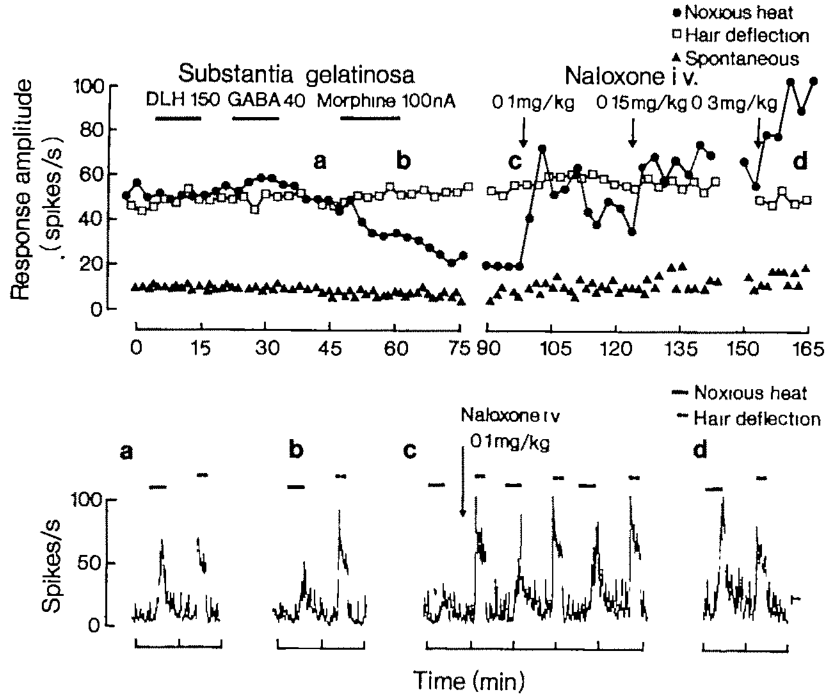

Figure 4 Depression by morphine, ejected in the substantia gelatinosa, of nociceptor but not mechanoreceptor activation of a dorsal horn neurone and reversal of this effect by intravenous naloxone in the cat. This neurone was located in spinal lamina IV and was activated by noxious heat to the third digital pad of the left hind limb and by deflection of adjacent hairs; separation between drug administering and recording micropipettes, 230 µm. The lower tracings are pen recordings of cell firing; the upper record is a graph of response amplitude in spikes per second with respect to time for the noxious and innocuous stimuli. As the response to deflection of hairs consisted of an initial burst followed by a slow decline in cell firing, the firing frequency present mid-way through the stimulus period has been plotted. Spontaneous firing is also plotted. The bars above the graph indicate the times of ejection of DL-homocysteate (DLH), γ-aminobutyric acid (GABA) and morphine in the substantia gelatinosa. Ejection currents are in nanoamperes (nA). The letters a, b, c and d indicate the times at which the corresponding lower traces were recorded.

without effect on mechanoreceptor-induced firing is shown in Figure 4. Ejection of DL-homocysteate and GABA in the substantia gelatinosa had no effect on the firing of this neurone. Compared with control observations (Figure 4a), those recorded after 15 min of morphine ejection into the substantia gelatinosa (Figure 4b) show approximately a 40% reduction in firing to noxious heat but no change in the response to hair deflection. With this and other neurones, further depression of nociceptive responses occurred following cessation of morphine ejection (Figure 4c).

Figure 4 also shows that, on this neurone, spontaneous firing was not depressed concomitantly with nociceptor-induced firing, an observation also made with 9 other neurones (see Figure 6). With 4 cells, however, both spontaneous firing and nociceptive responses were reduced in parallel, a result illustrated in Figure 5. With the remaining neurone, the nociceptive responses of which were reduced by morphine, spontaneous firing was increased.

The time course of the effects of morphine ejected in the substantia gelatinosa showed differences from those usually observed when drugs are administered electrophoretically near cell bodies. Firstly, there was considerable variation in the time to onset of the reduction of nociceptive activation (4–35 minutes). Even when these times were corrected to effective doses of morphine (total charge in microcoulombs passed through the morphine containing barrel up to the time of reduction of nociceptive responses) the scatter was considerable: the mean effective dose was 102 µC ± 79 s.d. This scatter probably represents in part the variation in the positioning of the drug administering pipette with respect to the receptors affecting each neurone.

Secondly, the reduction of nociceptive activation by

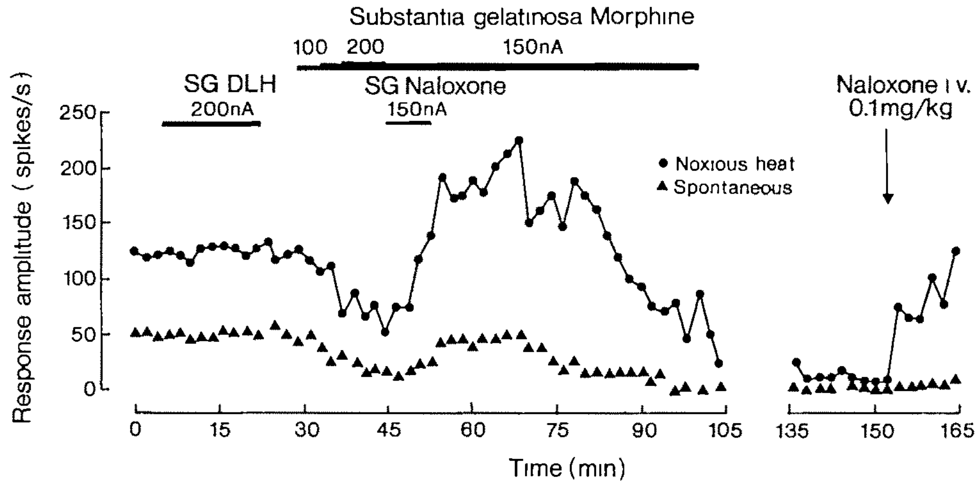

Figure 5 Prolonged effect of morphine, ejected in the substantia gelatinosa, in reducing nociceptive activation of a dorsal horn neurone and reversal of this effect by naloxone administered both electrophoretically and intravenously in the cat. Lack of effect by DL-homocysteate is also shown The neurone was located in spinal lamina IV and activated by noxious heat to the fourth digital pad of the left hind limb; separation of tips of drug administering and recording micropipettes 200 µm. Response amplitude and spontaneous firing are plotted with respect to time The times of ejection of DL-homocysteate (DLH), morphine and naloxone are indicated by bars above the graph.

morphine was long lasting but, because its abrupt reversal by intravenous naloxone was considered necessary evidence of specificity, the duration of action was not determined with any accuracy. In five experiments depression by morphine was still unchanged at 12, 17, 21, 50 and 56 min after cessation of morphine ejection into the substantia gelatinosa.

Figures 4 and 5 illustrate results from two such experiments. In each case both the prolonged control observations and the abrupt increase in firing by noxious heat which followed intravenous naloxone suggest strongly that morphine was still active in reducing nociceptive responses 40–50 min after cessation of ejection in the substantia gelatinosa.

(iii) Reversal by naloxone

(a) Electrophoretic naloxone. Naloxone, ejected electrophoretically with currents of 30–200 nA in the region of the substantia gelatinosa from the same pipettes used to administer morphine, reversed the depression of nociceptive responses by morphine on all 13 cells tested. In these experiments naloxone ejection was added to that of morphine, after the latter drug had reduced nociceptive responses, and morphine ejection was continued beyond that of naloxone until nociceptive activation had once more been reduced (Figure 5). There were two types of result. In the first (4 neurones) naloxone restored nociceptive responses to control levels with no significant effect on non-nociceptive responses or spontaneous firing. In the second group (9 neurones) naloxone not only antagonized the effect of morphine but enhanced nociceptive responses to values well above those of controls (Figures 5 and 6). This was accompanied by an increase in cell excitability as shown by increased spontaneous firing and non-nociceptive responses when the latter were tested (7 neurones). As Figure 6 shows, however, the increases in nociceptive responses were considerably greater than the changes in the other firing parameters.

On each neurone the ejecting current of naloxone necessary to antagonize the action of morphine was approximately equal to the current of morphine necessary to reduce nociceptive responses (see Figures 5 and 6). As Figure 5 illustrates, large ejecting currents of morphine for prolonged periods were required to reduce responses to levels similar to those present prior to naloxone ejection.

(b) Intravenous naloxone. When administered intravenously after morphine ejection was terminated, naloxone abruptly reversed the depression of nociceptive activation by morphine in 4 of 5 experiments. In three experiments the first dose of naloxone was effective (0.1, 0.3 and 0.3 mg/kg) and in the remaining one, two doses of 0.3 mg/kg were necessary. In 3 of these 4 experiments, subsequent doses of intravenous naloxone increased firing by

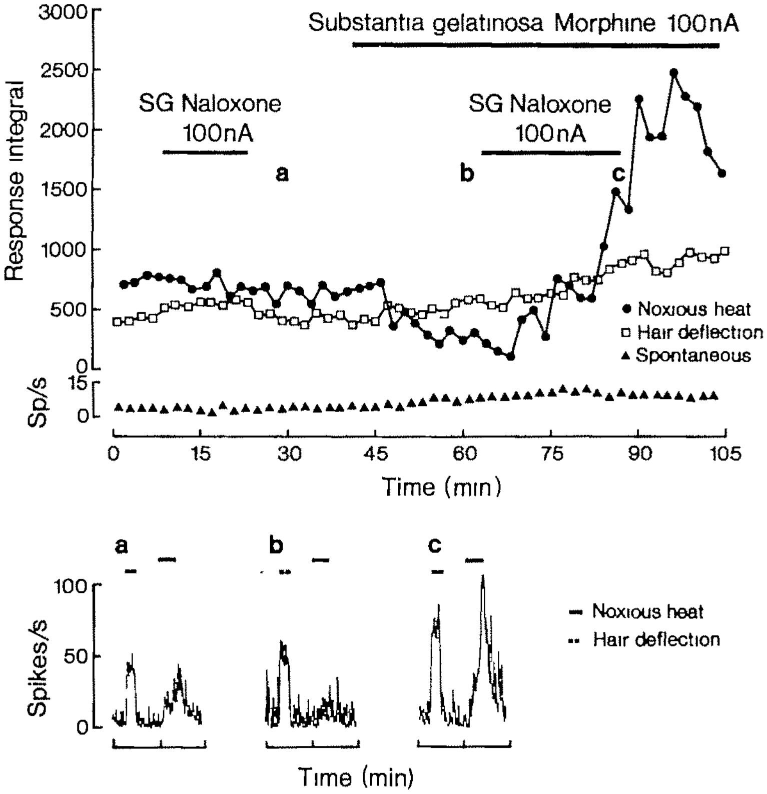

Figure 6 Contrasting effects of naloxone ejected alone in the substantia gelatinosa and naloxone ejected after morphine, on the firing of a dorsal horn neurone in the cat. This neurone was located in lamina IV and activated by noxious heat to the fifth digital pad of the left hind limb and deflection of adjacent hairs; separation between drug administering and recording micropipettes, 240 µm. Upper record – a graph of the total number of action potentials evoked by each stimulus (response integral) with respect to time. These counts have been corrected for spontaneous firing (gate duration for the noxious stimulus, 40 s, for the innocuous stimulus, 15 s). Spontaneous firing rate is also plotted. Lower tracings – cell firing measured with a rate meter The letters a, b and c refer to the times on the graph at which the records illustrated were observed

noxious heat to levels above those of control observations and this was accompanied by a lesser increase in spontaneous firing (Figure 4d).

Administered intravenously during the ejection of morphine, naloxone (0.5–1.2 mg/kg) antagonized the reduction of nociceptive responses by morphine in 3 of 6 experiments. All of these results were obtained from cells on which the effects of morphine had previously been antagonized by electrophoretic naloxone and on which prolonged ejection of morphine was necessary to reduce responses below control levels. In the experiments in which intravenous naloxone had no effect, the approximate amounts of morphine previously ejected were 150 nA for 90 min, 200 nA for 105 min and 200 nA for 150 minutes. In those in which intravenous naloxone reversed the effects of morphine the ejecting currents of morphine were 200 nA for 120 min, 30 nA for 70 min and 60 nA for 90 minutes.

(iv) Effects of naloxone alone. Naloxone, amongst the opiate antagonists, is said to exhibit the least agonist activity (Blumberg & Dayton, 1972). In two thirds of the present experiments, naloxone, administered electrophoretically after morphine, did not merely antagonize the effects of morphine but raised the three types of firing studied, spontaneous, nociceptive and non-nociceptive, above control levels (Figure 6). Hence it became necessary to test this substance for possible excitant effects *per se* when administered in the region of the substantia gelatinosa.

Twelve cells were studied in this way, 8 in lamina IV and 4 in lamina V.

Ejected with currents and times similar to those used to reverse the effects of morphine, naloxone had no effect on the firing of 9 neurones. With 2 cells the three parameters (spontaneous firing and the responses to both noxious and innocuous skin stimuli) were elevated just above control values during naloxone ejection and with one cell a similar effect was observed on spontaneous and nociceptive-induced firing, the only two firing patterns studied.

In no instance were the effects of naloxone, administered alone, similar to those observed when this substance was given after morphine. Figure 6 illustrates these differing actions of naloxone on one neurone. In this experiment naloxone 100 nA for 14 min had no effect on cell firing. Ejected after morphine had reduced nociceptive responses, this dose of naloxone restored nociceptive responses to control values. Continued ejection increased nociceptive responses to levels well above controls.

(v) Effects of amino acids. In an attempt to gain some insight into the possble site and mode of action of morphine, excitant (L-glutamate, DL-homocysteate) and depressant (glycine, GABA) amino acids were also ejected from micropipettes positioned in the substantia gelatinosa. In contrast to morphine there was considerable variation in the effects on spontaneous firing and on the responses to noxious and innocuous stimulation.

Of the 15 cells on which morphine was subsequently shown to be effective in reducing nociceptive responses, an excitant amino acid was also ejected from the morphine administering pipette with 11; with 4 no effect on any firing pattern was observed even after ejections of up to 200 nA for 13 min (Figures 4, 5); with 2 the spontaneous firing was variably increased but the responses to peripheral stimulation were unaffected; with 3 spontaneous firing and nociceptive, but not non-nociceptive, responses were increased; with one all three types of firing were increased and with one both spontaneous firing and the response to nociceptive afferents were reduced. Three cells, the nociceptive responses of which were unaffected by morphine, were all excited by an excitant amino acid but there was no consistent effect on the responses to skin stimulation.

Glycine and GABA seldom had any effect. Of 4 cells on which morphine subsequently reduced the nociceptive response, these amino acids had no effect on 3 (maximum current 200 nA for 5 min) and depressed the spontaneous firing and both responses (the non-nociceptive response being reduced before the nociceptive) with one, after ejection at 30–100 nA for a total of 15 minutes. Of 11 other cells on which glycine or GABA was tested, effects were seen on only 2 cells, both with GABA: with one spontaneous firing alone was reduced, and with the other the spontaneous and the nociceptive response were reduced.

At no time was the specific reduction of nociceptive responses produced by ejection of morphine observed following the ejection of amino acids.

A further difference from morphine lies in the time course of action of the amino acids. Although the time to onset of any detectable action of amino acids was frequently very prolonged (in relation to the usual latencies when amino acids are ejected near cell bodies), the reversal from such actions following the termination of ejection invariably occurred within 2 minutes.

Discussion

As the most common effect of morphine, administered near cell bodies, was to excite cells and produce abnormalities in action potential configuration, and these effects were not antagonized by naloxone, it is probable that the results obtained from this method of administration have no relevance to the depression by systemic opiates of the responses of dorsal horn neurones to noxious skin stimuli (Besson *et al.*, 1973; Le Bars *et al.*, 1975, Jurna & Grossman, 1976). This is in disagreement with the results of Calvillo *et al.* (1974) and Zieglgänsberger & Bayerl (1976) but supports the findings of Dostrovsky & Pomeranz (1976).

By contrast, when ejected in the region of the substantia gelatinosa, the effect of morphine was characterized by selectivity in the sensory modality affected, long duration and reversal by naloxone administered either electrophoretically or intravenously. Since the spinal cord was transected in all experiments these actions of morphine are independent of supraspinal influences. That these effects are relevant to the action of morphine administered systemically is supported by the following considerations:

Sensory modality selectivity

In humans, analgesic doses of morphine are without significant effect on olfaction, vision, hearing, touch, vibration and two-point discrimination (Wikler, 1950; Kreuger, 1955).

At the level of the spinal cord, several studies in cats indicate that intravenous morphine-like compounds have a preferential effect in reducing responses to impulses in small diameter primary afferent fibres. Using a combination of noxious natural stimulation and electrical stimulation of large diameter cutaneous afferents, Le Bars *et al.* (1975) found that morphine, 2 mg/kg, depressed the responses of lamina V neurones to the noxious stimulus alone. A similar result has been observed with phenoperidine (Besson *et al.*, 1973). Fentanyl (20–40 mg/kg) suppresses the discharge of spinal interneurones by impulses in Aδ but not in larger diameter fibres (Iwata & Sakai, 1971). Experiments on spinal reflexes have indicated that intravenous morphine preferentially reduces those reflexes produced by impulses in small diameter afferents (Wikler, 1945; Takagi, Matsumura, Yanai & Ogiu, 1955; Koll *et al.*, 1963; Krivoy, Kroeger & Zimmermann, 1973).

The present experiments, by demonstrating that morphine administered in the region of the substantia gelatinosa was selective in reducing firing by nociceptive but not by non-nociceptive afferents, thus show good correlation with the effects of systemic morphine. This selectivity is unlikely to be due to failure of morphine to reach terminals of mechanoreceptor afferents for although these fibres establish contacts with dorsal horn neurones outside the substantia gelatinosa, the terminals of both large diameter and small diameter primary afferent fibres are widely distributed within the substantia gelatinosa (Cajal, 1909; Ranson & Billingsley, 1916; Szentágothai, 1964; Scheibel & Scheibel, 1968).

Antagonism by intravenous naloxone

When substances are administered electrophoretically, tissue concentrations are unknown and hence antagonism of the effects of morphine by electrophoretically administered naloxone may not be relevant to the action of systemic morphine. However, reversal of the effects of electrophoretic morphine by doses of intravenous naloxone as low as 0.1 mg/kg suggests that the concentrations of morphine in the substantia gelatinosa were not inappropriate to those present after analgesic doses of morphine administered intravenously to cat (Jurna & Grossman, 1976) and dog (McLane & Martin, 1967a).

When administered into the substantia gelatinosa after morphine, naloxone did not behave simply as an antagonist; rather, with most neurones, nociceptive responses were enhanced well above control levels and excitability was increased as shown by increased spontaneous firing and slightly enhanced responses to mechanoreceptor stimulation. As naloxone alone did not show such activity the effects of this compound were dependent on whether or not cells had previously been exposed to morphine. These findings parallel results obtained when morphine and naloxone are administered systemically. Given alone in doses of 0.05–0.1 mg/kg naloxone has a negligible effect on the firing of lamina V neurones and ventrolateral tract axons in response to stimulation of Aδ and C fibres, but, when given after morphine, responses depressed by morphine are enhanced above control values (Le Bars *et al.*, 1975; 1976; Jurna & Grossman, 1975).

The long duration of the effect of morphine administered in the region of the substantia gelatinosa differs from any other report of an effect of this substance given electrophoretically (Dostrovsky & Pomeranz, 1973; Bramwell & Bradley, 1974; Calvillo *et al.*, 1974; Satoh, Zieglgänsberger, Fries & Herz, 1974; Davies & Duggan, 1974; Gent & Wolstencroft, 1976). When neurophysiological parameters are measured, intravenous morphine is not a short acting drug (McLane & Martin, 1967b; Herz & Teschemacher, 1971; Le Bars *et al.*, 1975). A prolonged analgesic action of morphine is observed after intraventricular administration (Herz & Teschemacher, 1971) and has been attributed to the difficulty with which the hydrophilic morphine molecule penetrates nervous tissue. In the present experiments other processes such as a slow rate of removal from the substantia gelatinosa or a slow dissociation from receptors may have been responsible for the prolonged action of morphine.

The anatomical localization of the opiate receptor studied in the present experiments is not known. Autoradiographic studies in the rat (Pert, Kuhar & Snyder, 1975) have found the substantia gelatinosa to be an area rich in opiate receptors. Binding studies in monkey (Lamotte, Pert & Snyder, 1976) have found a similarly high level of opiate receptors in the upper dorsal horn (which includes the substantia gelatinosa). Both the distribution of silver grains in the former study and the results of dorsal root section in the latter have suggested a location of opiate receptors on structures other than cell bodies, a finding consistent with the results of the present experiments.

Because of the complexity of organization of the substantia gelatinosa any of the following mechanisms could explain the effects of morphine in the present experiments: (1) Inhibition of transmitter release from the terminals of nociceptive afferents. This could be produced by block of conduction in fine terminals or by activity at axo-axonic synapses known to be abundant in the substantia gelatinosa (Réthelyi & Szentágothai, 1969). (2) Antagonism of the postsynaptic action of the transmitter released by nociceptive afferents. The relevant receptors could be located on the dendrites of neurones of lower spinal laminae or on neurones of the substantia gelatinosa. (3) Depression of excitatory interneurones or excitation of inhibitory interneurones of the substantia gelatinosa, although the inactivity of amino acids when ejected in this region may exclude this explanation.

The importance of the present experiments lies in the demonstration that morphine, ejected in the region of the substantia gelatinosa, has effects that can be related to analgesia induced by systemic opiates. Because of this, the technique offers a means of investigating the physiological role, if any, of the endogenous polypeptides (Hughes, 1975) which compete with morphine for stereospecific receptor sites in brain homogenates (Simantov, Kuhar, Pasternak & Snyder, 1976).

The authors wish to thank Miss A. Lind and Mrs M. Rodda for technical assistance. Naloxone was a gift from Endo Laboratories, New York. P.M.H. is a Queen Elizabeth II Fellow.

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(Received December 10, 1976.
Revised March 8, 1977.)

Br. J. Pharmac. (1977), 61, 77–82

THE ENHANCEMENT OF CHLORPROMAZINE-INDUCED HYPOTHERMIA BY LESIONS IN THE ANTERIOR HYPOTHALAMUS

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1 Administration of chlorpromazine (Cpz), either systemically or centrally, to unanaesthetized rats at an environmental temperature of 23 °C caused dose-dependent hypothermia.

2 In order to achieve equivalent hypothermia, intraventricular administration required a total dose of 20 µg Cpz and intraperitoneal administration a dose of 9.7 mg/kg body weight. Accordingly, the dose-ratio between intraventricular and intraperitoneal administration was 1 to 110. Cpz apparently exerts its hypothermic effect by acting directly on central nervous structures rather than through peripheral sites.

3 Cpz-induced hypothermia was potentiated by preoptic anterior hypothalamic (POAH) lesions but not by lesions of the ventromedial nucleus (VMN) of the hypothalamus. It was found that Cpz induced hypothermia most readily in rats with large POAH lesions (−10.4 °C), less so in rats with spinal lesions (−5.5 °C) at least with control rats (−2.9 °C).

Introduction

Chlorpromazine (Cpz), a major tranquillizer, produces a fall in body temperature when administered systemically to man (Ayd, 1955) and animals (Kopera & Armitage, 1954; Dobkin, Gillert & Lamoureaux, 1954; Hoffman & Zarrow, 1958) in a neutral thermal environment. Because of its hypothermic effect, the drug has been proposed as an adjunct in the production and maintenance of hypothermia (Gray & Graham, 1971) and in the treatment of heat stroke (Hoagland, 1961) in clinical medicine. However, the site and mechanism of this action of Cpz is not clear. It could act on peripheral thermal elements, on the temperature regulating structures within the CNS, or on both.

In the rat, Cpz-induced hypothermia is due, in part, to increased peripheral vasodilatation (Kollias & Bullard, 1964), increased surface area by postural change (Le Blanc, 1958), decreased oxygen consumption (Courvoisier, Fournel, Ducrat, Kohlsky & Koetschet, 1953; Hoffman, 1958; Kollias & Bullard, 1964), and loss of piloerection and shivering (Kollias & Bullard, 1964). Most of these investigators have suggested that the hypothermic effect following systemic injection of Cpz is due to an action on central thermoregulating structures. Nevertheless, conflicting reports exist on the effect of intrahypothalamic injections of Cpz on the body temperature of the rat. Unexpectedly, a dose-dependent hyperthermia was seen after central administration of the drug (Rewerski & Jori, 1968; Kirkpatrick & Lomax, 1971; Rewerski & Gumulka, 1974). Kirkpatrick & Lomax (1971) even concluded that the hypothermia induced by systemically administered Cpz is mediated by sites outside the CNS. They discovered that systemic administration of *N*-methyl Cpz (a quarternary analogue of Cpz), a substance which supposedly has difficulty penetrating the blood-brain barrier, also produces hypothermia. On the other hand, direct injection of this drug (Cpz) into the preoptic anterior hypothalamus (POAH) has been shown to produce dose-dependent hypothermia in monkeys (Chai, Fann & Lin, 1976) and in hamsters (Reigle & Wolf, 1971).

The present study was undertaken to elucidate the possible locus of Cpz-induced hypothermia in rats by means of two different approaches: (1) The effects of surgical intervention of the POAH area and the spinal cord, the two proposed thermoregulating structures (Lin & Chai, 1974; Simon, 1974), on the hypothermia induced by systemically injected Cpz were determined. (2) The order of effectiveness of Cpz administered by various routes (namely, peritoneum, jugular vein, internal carotid artery, and 4th and 3rd cerebral ventricle) on body temperature was determined.

Methods

One hundred male Sprague-Dawley rats, with body weights between 230 and 300 g at the time of surgery, were used. The rats were housed individually in wire-mesh cages in a room of 25 ± 1.0°C with natural light-dark cycles. The rats were given free access to tap water and granular young-chicken feed supplied by the Taiwan Sugar Corporation. Food and water intake, body temperature, and body weight were measured daily at 08 h 00 min. During the experiments, the rats were placed in a prone position on a board. Ambient temperature during the experiments was 23 ± 0.5°C.

Surgical intervention

Each rat underwent either ablation of CNS tissue or chronic cannula implantation in an aseptic surgical operation under general anaesthesia of pentobarbitone sodium (6 mg/100 g, i.p.). Details of the methods used to prepare chronic rats with lesions in POAH or the spinal cord have been described previously (Lin & Chai, 1974). In brief, electrolytic destruction of POAH was accomplished bilaterally with anodal d.c. current of 2 mA for 20 seconds. The stereotaxic coordinates used were 8.5 mm anterior to the ear bars, 0.8 mm lateral to the midline, and 0.8 mm above the base of the skull. In preparing spinal animals, the cervical vertabrae was exposed and complete transection was made with a spatula at the seventh cervical spinal cord (C7). Ablation of the ventromedial nucleus of the hypothalamus (VMN) was performed according to the technique of Liu & Yin (1974). The stereotaxic coordinates used were 6 mm anterior to the ear bars, 0.5 mm above the base of the skull, and 0.5 mm left or right to the midline.

Implanation of unilateral cannula in the 4th or 3rd cerebral ventricle followed methods described previously (Chai, Chen & Yin, 1971; Lin & Chai, 1972). A 22-gauge guide tube was chronically implanted into the ventricle. The drug was administered through a 10 μl Hamilton microsyringe with the tip of a 27 gauge tube passing just beyond the outlet of the guide tube. Implanation of intrajugular and intracarotid catheters was based on techniques of Corbit (1965) and Chai, Lin, Chen & Wang (1971), respectively. The left jugular vein or the left internal carotid artery was selected for chronic cannulation. Intraperitoneal injections were made by puncture through the abdominal wall. A 1 ml syringe was used for the intrajugular, intracarotid, and intraperitoneal injections.

A period of 2–6 weeks was allowed to permit the animals to recover from the surgical operation before they were used for experiments. For rats with POAH lesions or spinal transection, gradual recovery of appetite (food consumption greater than 10 g per day), an increase in body weight, and a normal range of body temperature (maintained under room temperature) were good indications of general recovery. During the control period of observation in an ambient temperature of 23°C, animals showing a rectal temperature below 37°C, above 38.9°C, or great fluctuations were excluded.

Following the experiments, the rats were killed by an overdose of pentobarbitone sodium and the heads were perfused with 10% formalin saline. The brains were removed, cut in 40 μm sections, and stained with thionin to verify the site of the brain lesions or spinal transection and the tips of the cannulae for intracerebroventricular injection. The exact location of intrajugular and intracarotid cannula was verified by gross inspection.

Drug injection

Chlorpromazine hydrochloride (supplied by Smith Kline & French Laboratories) was added to pyrogen-free distilled water just before use and adjusted to pH 7.0–7.3 in order to make the various molar solutions. The volume and the molar concentration of Cpz solutions used in different routes of injection were as follows: intraperitoneal, intravenous and intracarotid injections of 5–15 mg/kg, 2–6 mg/kg and 0.1–0.5 mg/kg Cpz, respectively, were given in 1 ml 0.9% w/v NaCl solution (saline)/kg body weight. On the other hand, fourth and third cerebroventricular injections of 10–40 μg Cpz for each route were given in 5 μl saline per animal. Control injections of pyrogen-free sterile NaCl solutions equal in volume and molarity to the Cpz solutions were given by the same routes. All the solutions used for injection were prepared in pyrogen-free glassware which was baked at 180°C for 4 h before use.

Temperature recordings

Rectal temperature was monitored with a Tri-R-6 flexible thermistor probe, and the subcutaneous temperature of the tail was monitored by a Tri-R hypodermic needle thermistor. Respiratory movements were monitored with a pneumograph, connected to a Statham SP37 transducer. All recordings were made continuously on a Grass polygraph. Shivering-like movements were observed grossly.

Results

Injections of Cpz, given either systemically or centrally at an ambient temperature of 23°C caused dose-dependent decreases in rectal temperature, varied degrees of psychomotor depression, an initially higher rate of respiratory frequency, and an initially higher tail skin temperature (subcutaneous vasodilatation). Changes in rectal temperature were the only responses measured specifically.

Responses to thermal exposure and effects of intraperitoneal administration of chlorpromazine on lesioned animals

In this series of experiments, tests of thermal exposure preceded Cpz injection by two days. Figure 1 and Table 1 show maximum changes in rectal temperature of intact and spinal rats, rats with POAH lesions, and rats with VMN lesions after 1 h of heat (36°C) or cold (8°C) exposure. They also show the decrement in the rectal temperature of these animals in response to Cpz administration at an ambient temperature of 23°C. Abrupt exposure to heat produced significant hyperthermia, subcutaneous vasodilatation and hyperpnoea, whereas exposure to cold produced the opposite effects, marked hypothermia, subcutaneous vasoconstriction and bradypnoea. Figure 1 shows that the rise or fall in rectal temperature of rats with lesions in POAH (+3.9°C in the heat; −6.5°C in the cold) and spinal rats (+2.6°C; −4.4°C), upon heat or cold exposure, were significantly higher than those of control rats (+1.5°C; −3.0°C) and rats with lesions in VMN (+1.6°C; −3.2°C). Hence, lesions in POAH or in the cervical spinal cord (at C7) caused a thermoregulatory deficit against heat and cold. Moreover, the extent of the thermoregulatory deficit was related to the sites and size of the brain lesions. Large lesions in POAH produced more marked deviations of the rectal temperature during thermal stress (+3.9°C in the heat vs −6.5°C in the cold) than did small lesions (+2.8°C; −4.5°C). Figure 2 shows outline drawings of brain sections taken from 2 rats with large lesions in POAH (a–c and d–f) and 1 rat with small POAH lesions (g–i). On the other hand, the animals with lesions in VMN tolerated heat or cold stress as well as did the control animals.

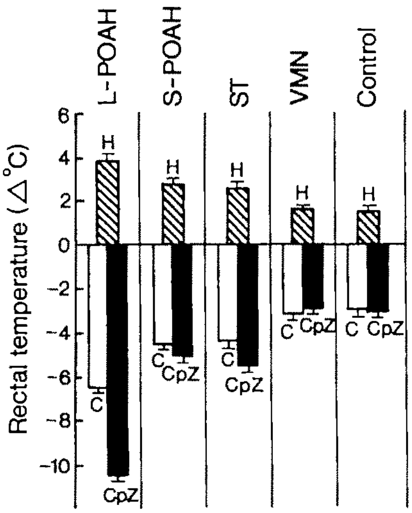

Figure 1 Temperature responses to external heat (36°C) and cold (8°C) exposure, and to an intraperitoneal dose of chlorpromazine (Cpz) (10 mg/kg; ambient temperature of 23°C) in control rats and rats with lesions in preoptic anterior hypothalamus, ventromedial nucleus of hypothalamus and spinal cord. L-POAH: large lesions in preoptic-anterior hypothalamus; 8 animals. S-POAH: small lesions in preoptic-anterior hypothalamus; 4 animals. ST: spinal transection at C7; 4 animals. VMN: lesions in ventromedial nuclei of hypothalamus; 4 animals. Control: 8 intact animals. H: heat exposure. C: cold exposure.

Table 1 Changes of rectal temperature in response to an intraperitoneal dose of chlorpromazine in control animals and in animals with lesions in the preoptic anterior hypothalamus (POAH), the ventromedial nucleus of the hypothalamus (VMN) and the spinal cord at an environmental temperature of 23°C

No. of animals	*Treatment of animals*	*Route injection and dose*	*Temperature decrement*			
			Latency	*Maximum*	*Time to maximum*	*Recovery time*
		(mg/kg)	*(min)*	*(°)*	*(min)*	*(min)*
8	Control	i.p., 10	15±2.36	3.1±0.24	96± 4.88	440±16.65
8	L-POAH	i.p., 10	6±1.82	10.4±0.38*	390±10.25	2280±50.75
4	S-POAH	i.p., 10	9±1.22	5.1±0.26*	180±12.53	270±35.12
4	ST	i.p., 10	12±1.02	5.5±0.31*	210±12.78	900±42.51
4	VMN	i.p., 10	15±2.20	2.9±0.28	150±10.45	500±18.35

L-POAH: large lesions in preoptic-anterior hypothalamus; S-POAH: small lesions in preoptic-anterior hypothalamus; ST: spinal transection at C7; VMN: lesions in ventromedial nuclei of hypothalamus. Values are means ± s.e.

* Indicates statistically significant change with P values < 0.05 calculated from Student's t test.

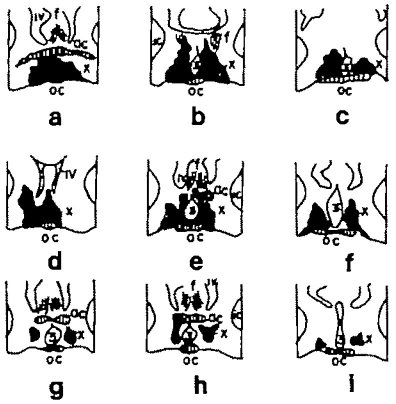

Figure 2 Drawings of photomicrographs of brain sections taken from 2 rats with chronic large lesions in preoptic-anterior hypothalamus show sites and size of lesions from rostral to caudal portions (a–c and d–f) and 1 rat with small lesions in POAH (g–i). f: fornix; ac: anterior commissure; 3: 3rd cerebral ventricle; IV: 4th cerebral ventricle; ic; internal capsule; oc: optic chiasma; x: sites of lesions.

The magnitude of the hypothermic effect of Cpz (10 mg/kg, i.p.) also depended on the location as well as the size of the lesions. The order of effectiveness of Cpz-induced hypothermia decreased from the rats with large lesions in POAH (10.4°C maximum decrement in rectal temperature) to the spinal rats (5.5°C maximum temperature decrement) and finally to the control rats (2.9°C maximum temperature decrement). Other parameters, e.g., latency, time to maximum temperature decrement, and recovery time, correlated well with the magnitude of temperature decrement produced by Cpz (see Table 2 for details).

Effect of chlorpromazine on body temperature of normal rats as related to dose and route of administration

Table 2 shows the hypothermic effect of Cpz administered via the peritoneum, femoral vein, internal carotid artery, and fourth and third cerebral routes at an ambient temperature of 23°C. It was found that the dose required to produce a drop of around 3°C in rectal temperature decreased as the route varied from central to systemic, i.e., from an intracerebroventricular route (20 µg in total) to an intracarotid route (0.25 mg/kg) and finally to an intraperitoneal route (9.75 mg/kg) of administration. In other words, direct injection of small amounts of Cpz (20 µg in total) into the cerebral ventricle produced the same hypothermic effect (3°C) as intraperitoneal injection of a large dose (9.75 mg/kg), a ratio of 1 to 110. This

Table 2 Hypothermic effect of chlorpromazine administered via peritoneum, femoral vein, internal carotid artery, and fourth and third cerebral ventricle routes at an environmental temperature of 23°C

			Temperature decrement			
No. of animals	*Route of administration*	*Dose*	*Latency* *(min)*	*Maximum* *(°C)*	*Time to maximum* *(min)*	*Recovery time* *(min)*
4	Peritoneum	5 mg/kg	18±2.28	2.4±0.23	62± 4.74	180± 9.26
4	Peritoneum	10 mg/kg	14±2.10	3.3±0.23	90± 5.64	430±15.32
4	Peritoneum	15 mg/kg	6±1.02	4.5±0.35	130± 6.37	460±17.43
4	Femoral vein	2 mg/kg	15±3.54	3.1±0.20	85± 6.32	410±14.28
4	Femoral vein	4 mg/kg	10±1.64	3.9±0.32	92± 8.25	510±17.56
4	Femoral vein	6 mg/kg	7±1.12	4.6±0.32	115± 8.61	550±20.12
4	Internal carotid artery	0.1 mg/kg	6±1.25	2.2±0.30	149± 7.47	590±20.10
4	Internal carotid artery	0.25 mg/kg	6±1.21	2.8±0.31	155± 8.62	610±22.30
4	Internal carotid artery	0.5 mg/kg	5±0.25	3.8±0.33	211±20.86	650±25.62
4	4th cerebroventricle	10 µg	5±0.19	2.6±0.19	68± 5.24	380±16.21
4	4th cerebroventricle	20 µg	3±0.27	2.9±0.19	270±18.75	520±16.32
4	4th cerebroventricle	40 µg	2±0.24	3.8±0.15	377±23.60	740±22.56
4	3rd cerebroventricle	10 µg	6±0.22	2.5±0.21	71± 5.21	370±13.32
4	3rd cerebroventricle	20 µg	4±0.18	3.2±0.29	160± 8.46	480±16.34
4	3rd cerebroventricle	40 µg	2±0.21	4.2±0.34	362±17.45	760±21.63

Values are means ± s.e.

suggests that chlorpromazine exerts its effect mainly through the CNS. However, there was no significant difference in the Cpz-induced hypothermia between the routes of administration of 3rd and 4th cerebral ventricles in the range of doses being tested.

Although psychomotor depression occurred in all rats immediately after Cpz administration, some recovered much more rapidly than others. For instance, some showed rearing locomotion only 0.5 to 1.0 h after administration. However, there was no relation between recovery from psychomotor depression and degree or duration of hypothermia. Furthermore intracerebroventricular administration of Cpz induced marked hypothermia, yet there was little apparent psychomotor depression.

Administration of Cpz produced a temporary increase in subcutaneous temperature and respiratory frequency. These reactions were concomitant with the reduction of rectal temperature. However, as the rectal temperature fell 1.5 to 2.0°C, the tail temperature dropped rapidly as did the respiratory frequency. By the time the hypothermia reached its maximum, the tail temperature and the respiratory rate had returned to the resting level or even lower.

Discussion

In the present study, the most striking finding was the high correlation between the extent of thermoregulatory deficit and magnitude of Cpz-induced hypothermia. Lesions in POAH or spinal transection at C7 caused both a thermoregulatory deficit against heat and cold and an enhancement of Cpz hypothermia, compared to that seen in control animals and animals with VMN lesions. Animals with large lesions of the POAH region had a larger thermoregulatory deficit than those with small lesions in the POAH or those with spinal transection at C7. This was revealed by the changes in body temperature of groups of animals with these lesions when they were exposed to thermal stress.

The present study was concerned with the additional question of whether Cpz caused changes in body temperature by acting directly on the central or peripheral control system. For an equivalent hypothermia (−3.0°C), the effectiveness of Cpz decreased from intracerebroventricular to the intracarotid and intravenous and finally to the intraperitoneal route of administration. Hence, Cpz apparently exerts its hypothermic effect mainly through the central nervous system rather than through peripheral sites. Recent evidence has suggested that the temperature regulating system may include central nervous structures other than POAH, e.g., medulla oblongata and spinal cord (Hellon, 1971; Chai & Lin, 1973; Lin & Chai, 1974; Simon, 1974). Indeed, two distinct structures in the brain stem, namely, the POAH and medulla oblongata, have been found responsible for the Cpz hypothermia (Chai *et al.*, 1976). Furthermore, present results also showed a dose-dependent hypothermia in response to the direct injections of Cpz into third or fourth cerebral ventricle. The above data, taken together, imply that the induction of Cpz hypothermia may be attributed to a specific action of Cpz on these central thermoregulatory mechanisms in the POAH and the medullary areas.

In considering the question of the enhancement of Cpz-induced hypothermia in animals with brain lesions, the fact has to be considered that the extent or intensity of Cpz-induced hypothermia is related to the compensatory activity of the central thermoregulatory mechanisms. In intact animals, an intraperitoneal dose of 10 mg/kg of Cpz produced a maximum fall of 3.1°C in body temperature in an environment of 23°C. A displacement of 3.1°C from normal core temperature should be powerful enough to activate the various thermoreceptors in the body. The activated thermoreceptors, particularly those located in the central nervous system, then send feedback inputs (temperature-dependent) to the central control mechanisms to generate a series of compensatory reactions (e.g., vasoconstriction, bradypnoea, piloerection and shivering; Hellon, 1971). Under normal circumstances, these cold-induced compensatory reactions should counteract the Cpz-induced thermal responses and prevent further reduction of the body temperature. As a consequence, in animals with lesions in POAH and in spinal animals, the absence or reduction in the numbers of hypothalamic or spinal thermoreceptors would induce little compensatory reaction to counteract the massive Cpz-induced hypothermia and, therefore, would allow a severe reduction in body temperature, 10.4°C and 5.5°C, respectively. In addition, if we accept the phylogenetic hypothesis of 'cephalization' (that temperature regulating mechanisms that exist along the whole CNS become more concentrated in its anterior portions; Thauer, 1973) then, the present study showing that animals with large lesions in POAH underwent a more marked Cpz-induced hypothermia than spinal animals could be explained.

Finally, it should be mentioned that, in the present study, intracerebroventricular administration of Cpz induced marked hypothermia, yet there is little apparent psychomotor depression. This seems to indicate that a sedative effect on the animal is not a prerequisite for the Cpz hypothermia. The same suggestion has been made by Borbely, Huston & Baumann (1973).

This work was supported by the grants from National Science Council (Republic of China), J. Aron Charitable Foundation (U.S.A.) and Fogarty International Center (N.I.H., U.S.A.). The authors are grateful to Drs J.D. Hardy, W.S. Cain and Y. Nishi (of John B. Pierce Foundation Laboratory, Yale University, New Haven, Connecticut) for their constructive criticism during the preparation of this manuscript.

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*(Received January 12, 1977.
Revised March 22, 1977.)*

Br. J. Pharmac. (1977), **61**, 83–86

DO CENTRAL DOPAMINE RECEPTORS HAVE A PHYSIOLOGICAL ROLE IN THERMOREGULATION?

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1 Core and tail skin temperature was measured in rats which had guide cannulae implanted into their brains to allow drug injections directly into the preoptic anterior hypothalamus.

2 Apomorphine and dopamine (10 μg in 1 μl) injected into the area of the preoptic anterior hypothalamus caused a fall in core temperature which was preceded by a rise in tail skin temperature.

3 The decrease in core temperature following central injection of either apomorphine or dopamine was significantly reduced by pretreating rats for 2 h with pimozide (0.5 mg/kg i.p.).

4 Bilateral intrahypothalamic injection of pimozide (0.5 μg in 1 μl) significantly reduced the hypothermic effect of systemic apomorphine (1.25 mg/kg i.p.).

5 Control rats placed 65 cm below a 250 W infrared lamp responded with vasodilatation of tail skin blood vessels as indicated by an increase in tail skin temperature. Pimozide pretreatment (0.5 mg/kg i.p.) significantly reduced this response.

6 These results suggest that the preoptic anterior hypothalamus contains dopamine receptors which mediate hypothermia in rodents and raise the possibility that endogenous dopamine has a physiological role in thermoregulation.

Introduction

Since the original observation of Feldberg & Myers (1963), a great deal of attention has been paid to the possible role of hypothalamic noradrenaline and 5-hydroxytryptamine in the control of body temperature. In contrast, dopamine has been less well investigated and before 1971 only small or inconsistent changes in body temperature had been recorded after intraventricular or intracisternal injection of the amine (Brittain & Handley, 1967; Myers & Yaksh, 1968; Bruinvels, 1970). The advent of specific agonists and antagonists at the dopamine receptor significantly affected the work in this field when it was shown that intracerebroventricular injection of the specific agonist, apomorphine, produced hypothermia in the rat which was blocked by the specific antagonist, pimozide (Kruk, 1972). One possible reason for the failure of previous studies to reveal a similar effect for dopamine could be that it was inactivated before reaching the appropriate receptors. We have recently shown that in the rat the most responsive site to central injections of apomorphine was the preoptic anterior hypothalamus (Cox & Lee, 1977a) and as this is the probable location of the central thermostats (Myers, 1975), it seemed reasonable to question whether dopamine had a physiological function at this site.

Previous workers have presumed both a hypothalamic location of the dopamine receptors and that they have a physiological function (Kennedy & Burks, 1974; Quock & Gale, 1974). However, either the drug injections were made intracerebroventricularly so that the precise site of action was obscure or there was no attempt to test a physiological response (such as heat stress) so that no claim concerning the physiological role of the dopamine receptors could be made.

In this paper confirmation of a hypothalamic location for the dopamine receptors is presented and the effect of pimozide on the response of rats to an imposed heat load is investigated.

Methods

Male Sprague-Dawley rats weighing 250 to 300 g were used in all the experiments, which were carried out at an ambient temperature of 17 ± 1 °C.

Central injections

Stainless steel guide cannulae (0.5 mm external diameter) were implanted into the brains of rats anaesthetized with pentobarbitone (45 mg/kg i.p.) using a David Kopf Stereotaxic Frame according to the technique of Pellegrino & Cushman, 1967. The co-ordinates used, with bregma as the reference point, were anterior-posterior 1.8 mm, lateral 1.2 mm and depth 5.0 mm. Using these co-ordinates the tip of the guide cannula lay 3 mm above the desired point of injection in the preoptic region of the anterior hypothalamus. Drug injections were made seven days later via an injection cannula that was inserted into the guide cannula and that extended 3 mm past its tip. The dose volume of the central injections was standardized at 1 µl, injected over a 45 s period. After completion of the experiment the injection sites were verified histologically.

Temperature measurements

Core temperature was measured in lightly restrained rats with a rectal thermistor probe (L. Light Labs.) inserted to a depth of 4 cm. In experiments involving drug interactions, tail skin temperature was measured by a strap-on thermometer, attached to the base of the tail and insulated from the environment. In the heat load experiments, tail skin temperature was measured by a small surface thermistor probe of 4.0 mm diameter also lightly strapped to the base of the tail and insulated from the environment.

Heat load experiment

Rats were placed in restraining boxes 65 cm below a 250 W infrared lamp for 60 min and changes in core and tail skin temperature measured. An insulating panel protected the tail and the core and skin thermistors from the radiant heat source. Responses of control rats were compared with those of rats which had been injected 2 h earlier with pimozide (0.5 mg/kg i.p.).

Statistics

Comparisons between groups were made with the non-parametric Mann-Whitney U test and unless otherwise stated a significant difference between groups was taken as $P < 0.05$. For ease of comparison in all cases means ± s.e. are presented as the index of the response.

Drugs

Apomorphine hydrochloride (MacFarlan-Smith Ltd) and dopamine hydrochloride (Koch-Light Ltd) were freshly prepared in sterile 0.9% w/v NaCl solution (saline) containing 0.1% sodium metabisulphite as an antioxidant. A stock solution of pimozide (Janssen Pharmaceuticals) 10 mg/ml was made by dissolving 100 mg of the drug in 3 drops of glacial acetic acid and 3 drops of absolute alcohol before making up to a final volume of 10 ml with hot 5% glucose solution. Subsequent dilutions for injection were made in sterile saline immediately before the injection. Appropriate vehicle injected controls were run simultaneously. All doses refer to the free base.

Results

Central injections

Dopamine and apomorphine (10 µg in 1 µl) injected unilaterally into the preoptic area of the anterior hypothalamus caused a fall in core temperature in rats which was significantly different from controls ($P < 0.01$). In all cases an increase in tail skin

Table 1 Effect of pimozide on core temperature response of rats receiving intrahypothalamic (i.h.) or intraperitoneal (i.p.) injections of apomorphine and dopamine

Drug	*Dose*	*Route*	*Mean change in core temperature (°C ± s.e.)*	n
Saline	—	i.h.	+0.2 ± 0.1	7
Apomorphine	10 µg	i.h.	−1.0 ± 0.2*	8
Apomorphine + pimozide	10 µg 0.5 mg/kg	i.h. i.p.	+0.4 ± 0.2†	3
Dopamine	10 µg	i.h.	−0.7 ± 0.2*	11
Dopamine + pimozide	10 µg 0.5 mg/kg	i.h. i.p	+0.1 ± 0.1†	4
Saline + apomorphine	— 1.25 mg/kg	i.h. i.p.	−1.8 ± 0.3	5
Pimozide + apomorphine	0.5 µg 1.25 mg/kg	i.h. i.p.	−0.6 ± 0.3†	3

Means are for maximum change in core temperature occurring within 40 min of injection. Pimozide pretreatment time, 2 h for intraperitoneal and 15 min for bilateral intrahypothalamic injection.

* Significantly different from saline control, $P < 0.01$.

† Significantly different from appropriate agonist control $P < 0.05$ (Mann-Whitney U test).

temperature preceded the fall in core temperature, but the measurement of core temperature was more consistent and was therefore used in the expression of the results (Table 1). The effects of intrahypothalamic injection of dopamine and apomorphine were blocked by pretreating the rats with pimozide. Also injection of pimozide 0.5 μg bilaterally into the preoptic anterior hypothalamus significantly antagonized the effect of a systemic dose of apomorphine (Table 1).

Heat load experiments

Exposure of control rats to a radiant heat load resulted in vasodilatation of the blood vessels of the tail as indicated by a rapid rise in the tail skin temperature which had increased to 5.4 ± 0.25 °C within 60 min (Figure 1). In contrast, in rats pretreated with pimozide there was a slower rise in tail skin temperature, which had only increased by 2.9 ± 0.23 °C in the 60 min period. The differences between the control and pimozide pretreated groups were significant ($P < 0.01$) when tested by the Mann-Whitney U test. Core temperature in control rats rose slowly during exposure to the heat load so that it had increased by 1.03 ± 0.1 °C in 60 minutes. In pimozide pretreated rats, core temperature rose more rapidly so that it had increased by 1.75 ± 0.2 °C in the same 60 min period. The increase in core temperature in pimozide-treated rats was significantly greater than that in the controls ($P < 0.05$).

Discussion

The doses of dopamine and apomorphine used for either systemic or intrahypothalamic injection were chosen on the basis of previous experiments in which full dose-response curves were obtained (Cox & Lee, 1977a and b). Similarly the systemic dose of pimozide used was one which had previously been shown to be selective for a dopamine-mediated hypothermia (Cox & Lee, 1977a). In the drug interaction studies a strap-on thermometer was used, which would only give a qualitative measure of tail temperature. However, the fact that an increase in the tail skin temperature always preceded the fall in core temperature after intrahypothalamic injection of either apomorphine or dopamine suggested that it was the vasodilatation of the skin blood vessels which mediated the thermoregulatory events. An action of these drugs on dopamine receptors was indicated by the finding that systemic injection of pimozide, a selective dopamine antagonist (Anden, Butcher, Corrodi & Fuxe, 1970), completely blocked their effects. Further confirmation of both the site of action and the type of receptor came from the experiments involving bilateral intrahypothalamic injection of pimozide which significantly reduced the response to a systemic dose of apomorphine.

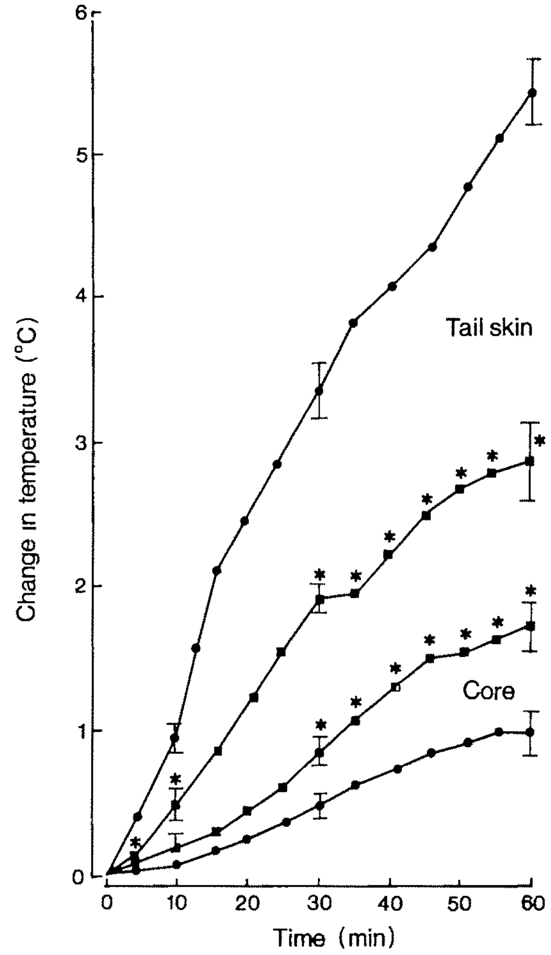

Figure 1 Change in core (lower two curves) and skin (upper curves) temperature of rats in response to an imposed heat load (250 W infrared lamp placed 65 cm above the lightly restrained rat) for saline (●) and pimozide (■) 0.5 mg/kg intraperitoneally pretreated rats. Each point is the mean of 4 separate observations; vertical bars indicate standard error. * Significantly different from saline control, $P<0.05$ Mann-Whitney U test.

If dopamine were indeed playing a physiological role in heat loss processes in rats, then pimozide injection might have been expected to have the opposite effect to dopamine and cause a hyperthermia. In our experiments neither systemic nor central injections of pimozide significantly changed skin or core temperature, thereby throwing doubt on the hypothesis. However, if the postulated dopaminergic heat loss system was not active at the relatively low ambient temperature of these experiments it was possible that pimozide would appear to be inactive. Therefore, a second type of experiment was carried out to assess the effect of dopamine receptor blockade on the response of rats to an imposed heat load. In these experiments a thermistor rather than a strap-on thermometer was used to provide a more reliable estimate of tail skin temperature. The fact that pimozide significantly reduced the ability of the rats to withstand the heat load supports the hypothesis of a physiological function of the hypothalamic dopamine receptors.

Such a concept would help to explain some previously inexplicable findings. Thus, as early as 1968, Rewerski & Jori found that central injection of chlorpromazine produced a hyperthermia in rats maintained at an ambient temperature of 20°C and that the greatest response occurred after injection into the anterior hypothalamus. These authors at first attributed this finding to an interaction with noradrenaline but subsequent work discounted this possibility (Rewerski & Gumulka, 1969). A more reasonable explanation for the hyperthermia would be blockade of hypothalamic dopamine receptors, which are normally involved in a heat loss pathway. It also seems likely that this dopaminergic system is present in other species. Hypothalamic dopamine receptors which mediate hypothermia have been reported in the adult fowl and cat (Marley & Nistico, 1972; Quock & Gale, 1974). Studies on rostral hypothalamic neurones sensitive to peripheral temperature changes have failed to reveal any consistent relationship between response to temperature and to acetylcholine, noradrenaline or 5-hydroxytryptamine applied iontophoretically (Jell, 1974). However more recent studies have suggested that a more consistent relationship is obtained when dopamine is the amine used (Jell, personal communication).

In conclusion, although a number of hypothetical models have been advanced for the involvement of monoamines in central thermoregulatory systems, dopamine has not usually been implicated. There would now seem to be a strong case for including this amine in future models.

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*(Received February 14, 1977.
Revised March 11, 1977.)*

Br. J. Pharmac. (1977), **61**, 87–89

INHIBITION BY A STABLE ANALOGUE OF ADENOSINE TRIPHOSPHATE OF PLATELET AGGREGATION BY ADENOSINE DIPHOSPHATE

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1 In citrated platelet-rich plasma, freshly prepared from rabbit blood, the velocity of platelet aggregation was within limits proportional to the log of the concentration of added adenosine diphosphate (ADP).

2 Addition of either adenosine triphosphate (ATP) or its β,γ-methylene analogue inhibited aggregation similarly except that the analogue was about half as potent as ATP. β,γ-Methylene ATP also reversed the optical effects associated with the shape change of platelets very similarly to ATP itself.

3 As β,γ-methylene ATP is not a substrate for nucleoside diphosphokinase, these observations do not support the proposition that inhibition of aggregation by added ATP is due to its utilization by the nucleoside diphosphokinase of platelets.

Introduction

The mechanism by which adenosine diphosphate (ADP) induces blood platelets to aggregate is still unknown (for discussion, see Elliot & Knight, 1975). One recent suggestion is that the effect depends on a nucleoside diphosphokinase reaction at the platelet surface (Packham, Guccioni, Perry & Mustard, 1974; Mustard, Packham, Perry, Guccioni & Kinlouch-Rathbone, 1975) in which added ADP acts as an acceptor of a phosphate removed from a phosphoprotein by diphosphokinase in the platelet membrane; and that this removal results in conformational changes required to initiate aggregation. On this hypothesis, the inhibition of aggregation by adenosine triphosphate (ATP) (Born & Cross, 1963) and, less effectively, by other nucleoside triphosphates is accounted for by assuming that added ATP competes with the phosphoprotein as an alternate donor of phosphate to added ADP.

If this were so, a synthetic analogue of ATP, viz. β,γ-methylene ATP which is not acted upon by nucleoside diphosphokinases and other enzymes which dephosphorylate ATP (Moos, Alpert & Myers, 1960; Myers, Nakamura & Flesher, 1963; Hegyvary & Post, 1971; Yount, Ojala & Babcock, 1971; Kidder, 1973), should not inhibit aggregation by ADP. This paper shows, however, that this analogue of ATP inhibits aggregation by ADP similarly to and only a little less potently than ATP itself.

Methods

New Zealand white rabbits had their ears shaved and the marginal veins cut across. The freely-falling blood was mixed with one-tenth volume of 3.8% trisodium citrate in polythene tubes which were centrifuged at 150 *g* for 15 min at room temperature of about 22°C. The platelet-rich plasma was separated and kept at room temperature during the aggregation experiments. As the platelet concentrations in the plasmas were rather high, each plasma was diluted with isotonic saline to make the concentration of platelets 3.0×10^8 ml.

Change in shape and aggregation was quantified as maximal rates of increase in transmitted light in a Mark IV aggregometer (Michal & Born, 1971) on the addition of ADP in various concentrations to 1 ml samples of diluted platelet-rich plasmas. For determining inhibitory effects, ATP or its β,γ-methylene analogue was added 10 s before ADP. ADP and ATP were highly purified preparations from the Sigma Chemical Company. The β,γ-methylene ATP was a gift from Dr H.O.J. Collier of Miles Laboratories, Stoke Poges, Bucks.

Results

The results of experiments in which inhibition produced by ATP was compared with that produced

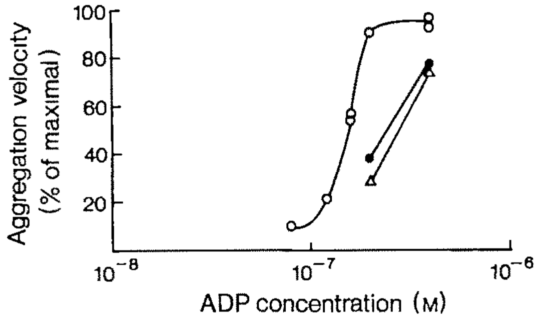

Figure 1 Inhibition of rabbit platelet aggregation by ATP or by β,γ-methylene ATP. Samples of rabbit citrated plasma (1 ml) diluted with saline to contain 3.0×10^8 platelets/ml were stirred at 900 rev/min in the aggregometer at 37°C. The points indicate aggregation velocities, as percentages of the maximal velocity, produced by different concentrations of ADP added alone (○) or 10 s after the addition of ATP 20 µM (●) or of β,γ-methylene ATP 40 µM (△).

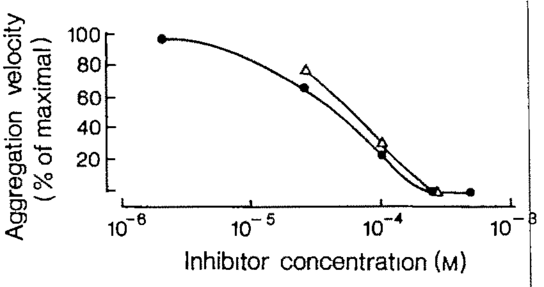

Figure 2 Inhibition of rabbit platelet aggregation by ATP or by β,γ-methylene ATP. Experimental conditions as for Figure 1 except that the plasma was diluted 1 in 5 with saline to contain 3.0×10^8 platelets/ml. The points indicate aggregation velocities produced by ADP 2 µM added 10 s after different concentrations of ATP (●) or of β,γ-methylene ATP (△). Values are expressed as percentages of maximal velocity produced by ADP 2 µM alone.

by β,γ-methylene ATP are shown in Figures 1 and 2. Whether the inhibitor concentrations were constant and the concentration of ADP was varied (Figure 1) or *vice versa* (Figure 2), the analogue inhibited aggregation in the same way as did ATP, although only about half as potently.

The rapid change in shape which platelets undergo on the addition of ADP can be reversed by the subsequent addition of ATP at a comparatively high concentration (Born, 1970). When ADP (2 µM) was added to rabbit platelet-rich plasma just after adding disodium edetate (EDTA, 6 nM) to prevent aggregation, the optical manifestation of the shape change of the platelets was reversed by β,γ-methylene ATP just as by ATP itself, although not quite so effectively (Figure 3).

Discussion

These observations indicate that an analogue of ATP which is not dephosphorylated by nucleoside diphosphokinase inhibits the shape change and

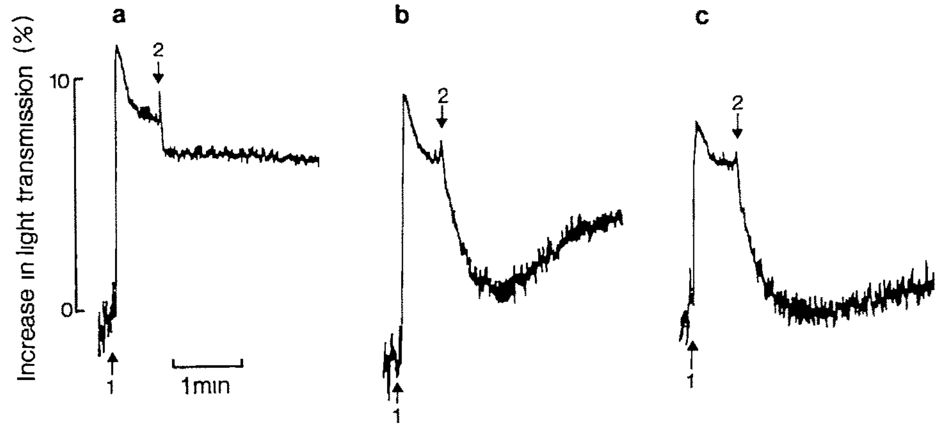

Figure 3 Reversal by ATP or by β,γ-methylene ATP of the increase in light transmission through platelet-rich plasma induced by ADP in the presence of EDTA To 1 ml samples of rabbit citrated plasma diluted 1 to 1.5 with saline to contain 3.0×10^8 platelets/ml and stirred in the aggregometer at 37°C was added EDTA 6 mM followed after 15 s by ADP 2 µM (arrow 1). Subsequent additions (arrow 2) were (a) saline, (b) ATP 1 mM or (c) β,γ-methylene ATP 1 mM

aggregation of platelets very much as ATP does. This result appears incompatible with the proposition that ATP must donate its terminal phosphate to produce inhibition of platelet aggregation by ADP. Furthermore, this analogue of ATP was a much more potent inhibitor than any of the naturally occurring nucleoside triphosphates (except ATP), all of which can be dephosphorylated by platelet nucleoside diphosphokinase (Packham *et al.*, 1974). There is good evidence (Macfarlane & Mills, 1975) that ATP causes inhibition by competing with ADP for a specific receptor on the platelet surface (Born, 1965). It seems likely that the β,γ-methylene analogue of ATP acts in the same way.

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(Received February 21, 1977)

Br. J. Pharmac. (1977), **61**, 91–96

HYPERTHERMIC EFFECTS OF MORPHINE: SET POINT MANIPULATION BY A DIRECT SPINAL ACTION

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1 Rats were implanted with chronic indwelling catheters in the lumbar spinal subarachnoid space in order to study the effects of morphine on body temperature.

2 Morphine administered intrathecally produced a dose-dependent rise in body temperature that was antagonized by naloxone.

3 The rise in body temperature evoked by a given dose of morphine appeared to be independent of the environmental temperature (4°C to 32°C) and was consistently associated with coordinated thermoregulatory responses (i.e. shivering and tail vasoconstriction). The fall in body temperature observed in these hyperthermic animals, following naloxone, was associated with a vasodilatation which coincided with the fall to the premorphine temperature level.

4 Morphine administered systemically also produced hyperthermia. This was only partially reversed by intrathecal naloxone. In animals made tolerant to the hyperthermic effects of systemic morphine, the intrathecal administration of naloxone produced a fall in body temperature.

5 Naloxone alone, administered either intrathecally or systemically, had no effect upon body temperature.

6 We suggest that morphine exerts a direct, pharmacologically specific effect on the spinal cord, which results in an altered thermoregulatory set point in the rat.

Introduction

The effects of morphine on body temperature have been shown to be complex, species-dependent and subject to experimental variables such as environmental temperature and restraint. In particular, work carried out in the rat has suggested that in the unrestrained rat, maintained in a high environmental temperature, systemically administered narcotics produce a rise in body temperature. In contrast, restraint and low environmental temperatures appear to attenuate the hyperthermic response or cause morphine to produce a frank hypothermia (Herrman, 1942; Gunne, 1960; Lotti, Lomax & George, 1965a & b; Paolino & Bernard, 1968; Martin, Dryzbylik & Spector, 1977). One suggested locus for this effect is the thermoregulatory centres associated with the anterior-preoptic region of the hypothalamus (Lotti *et al.*, 1965a & b; Foster, Jenden & Lomax, 1967; Lotti, 1973; Martin & Morrison, 1977). Intracerebral injection experiments have clearly demonstrated that opiates acting only within this delimited region can in fact exert a direct and pharmacologically specific effect (i.e. antagonized by naloxone or nalorphine) on the thermoregulatory response of several species including the rat (see above) and cat (Banerjee, Feldberg & Lotti, 1968). We now present evidence that the observed temperature response produced by systemically administered narcotics may also influence body temperature by a direct action on the spinal cord.

Methods

To study the local action of morphine in the spinal cord, rats under Nembutal anaesthesia were chronically implanted with catheters as described by Yaksh & Rudy (1976a). A polyethylene catheter was inserted into the spinal subarachnoid space through a slit in the cisternal membrane and extended to the rostral face of the lumbar enlargement. The distance from the point of insertion to the lumbar space was approximately 8.5 cm. The catheter was secured to the skull with screws and cranioplast cement. Experiments were started after a 7–10 day recovery period. All drugs injected intrathecally were dissolved in a volume of 10 µl of artificial cerebrospinal fluid. Each drug injection was immediately followed by an injection of 5 µl of the vehicle to clear the catheter.

All temperature experiments were carried out with the animal restrained in a hemicylindrical wire mesh cage with its tail protruding from the rear. Body temperature was measured by the insertion of a YSI 401 thermistor probe (Yellow Springs Instrument Co.), 6 cm into the colon. The probe was affixed to the tail by a loose wrapping of tape. In a number of experiments, tail temperature was also assessed as a measure of vasodilitation, with a specially constructed probe consisting of the normal YSI 401 thermistor to which had been attached the disc portion of a YSI 427 thermistor probe. When the colonic probe was in place, the disc probe lay with its surface on the base of the tail, approximately 2 cm from the rectum. All temperatures were continuously monitored on a potentiometric recorder. Experiments were normally carried out at an environmental temperature of 22°C, but in a limited number of experiments, the temperature response of the animal following intrathecally injected morphine was assessed in a high (32°C) and low (4°C) temperature environment. The doses referred to in this paper are given in terms of the weight of the salt of morphine sulphate (Merck) and naloxone hydrochloride (Endo).

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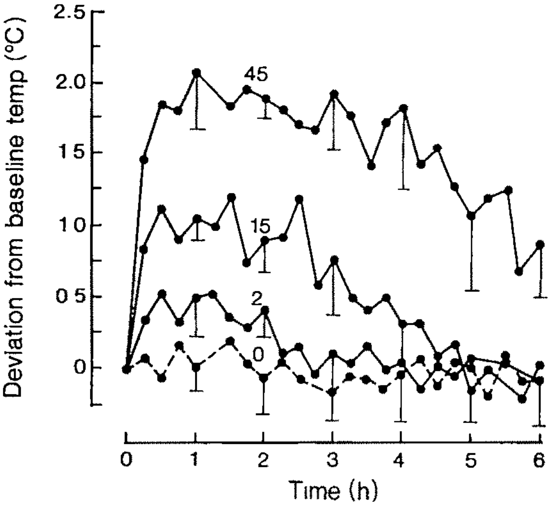

Figure 1 Mean deviation from baseline colonic temperature is plotted as a function of time after the intrathecal injection of morphine sulphate in doses of 0 to 45 µg. Each curve represents the mean response of from 4–6 rats. Vertical lines show s.e. mean.

Results

Effects of morphine in the spinal subarachnoid space

The injection of morphine into the spinal subarachnoid space of the rat produced a profound hyperthermia, the amplitude and duration of which was dose-dependent over a range of 2–45 µg. As shown in Figure 1, the hyperthermia was characterized by a relatively rapid onset at all doses, with the half maximum level of temperature rise being observed within 5–10 min after the injection. The injection of equal amounts of vehicle had no effect on the animal's body temperature. The intrathecal injection of morphine, at the doses employed, had no effect on heart rate, blood pressure or respiration and produced no signs of behavioural agitation. In view of the possibility that pyrexic effects produced by intracerebral injections may be associated with the release of prostaglandins, indomethacin was administered (5 mg/kg, i.p.) 2 h before the intrathecal injection of morphine (45 µg) in 4 rats. This amount of indomethacin, while inducing a slight hypothermia alone, had no effect on the observed morphine-induced hyperthermia.

Evidence for a set point change

To assess whether the hyperthermic action of morphine in the spinal cord resulted from a nonspecific blockade of heat loss responses or activation of heat gain mechanisms, the injections of morphine were carried out at three ambient temperatures (high, 32°C; thermoneutral, 22°C and low, 4°C). As well as colonic temperature, the tail temperature was recorded whilst the animals were closely observed for signs of tremor activity. Three experiments at each ambient temperature were carried out. The data presented in Figure 2 are the results obtained from one such animal and are representative of the results observed in the other two animals. At all ambient temperatures, intrathecal morphine (45 µg) produced a uniform rise in colonic temperature of approximately 2°C. The injection of morphine was uniformly associated with a fall in tail temperature at 32°C and 22°C ambient temperature, suggesting the initiation of an active vasoconstriction. At 4°C ambient temperature, the absence of any change in tail temperature suggests that the animal was already vasoconstricted. After approximately 55 min, naloxone was administered systemically (1 mg/kg, i.p.). Naloxone produced an immediate elevation in the tail temperature concurrent with the fall in colonic temperature. The rise in tail temperature, indicated active vasodilator control by the animal. As the body temperature approached premorphine levels, the tail temperature fell. This is illustrated in the record taken at 22°C. Though tremor activity, reflecting a heat gain response, was not quantified, the presence of such tremor was most apparent in all animals following the injection of morphine in the neutral and low temperature environments. While at the low temperature, tremor activity was observable in two animals before morphine; following its intrathecal injection the magnitude of the tremor activity was much enhanced.

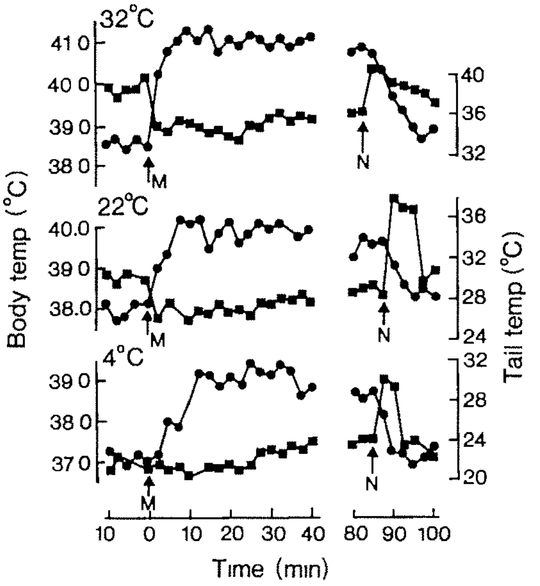

Figure 2 The rectal (●) and tail (■) temperature of a single rat is plotted as a function of time after the intrathecal injection of morphine sulphate (45 µg) at the first arrow (M) whilst the animal is in a hot (32°C), thermoneutral (22°C) or cold (4°C) environment. At the second arrow (N), the animal received an intraperitoneal injection of naloxone hydrochloride (2 mg/kg).

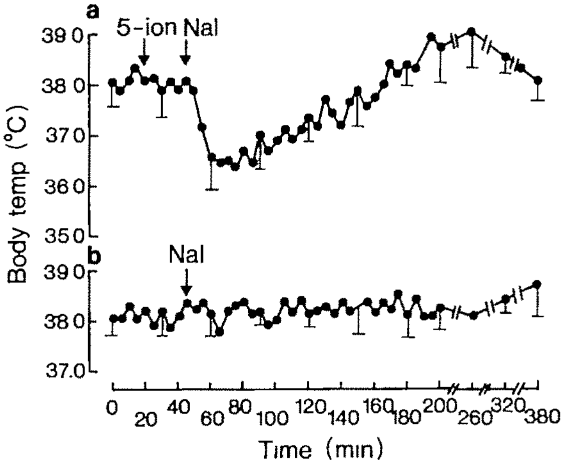

Figure 3 (a) Mean colonic temperature is plotted for 5 animals which had previously been rendered tolerant to the hyperthermic effects of systemically administered morphine. Vertical lines show s.e. mean. At the first arrow, a control intrathecal injection of vehicle (5-ion) was given, while at the second arrow, 10 µg of naloxone hydrochloride (Nal) was similarly injected. (b) The effect of an injection of naloxone (10 µg) on the colonic temperature of 4 control animals.

Table 1 Effects of intrathecally and systemically administered naloxone on the hyperthermia produced by systemically administered morphine

		Deviation from postmorphine baseline temp. (°C)	
Group	n*	*Post morphine*†	*Post naloxone*‡ *or vehicle*
Morphine + intrathecal vehicle	4	1.63 ± 0.42	1.78 ± 0.57**
Morphine + intrathecal naloxone (5 µg)	6	1.41 ± 0.34	0.63 ± 0.38§**
Morphine + intrathecal naloxone (50 µg)	4	1.72 ± 0.51	0.56 ± 0.24§**
Morphine + systemic naloxone (2 mg/kg)	4	1.58 ± 0.42	0.17 ± 0.32§

* Size of group; † Value is mean with s.e. mean, measured 45 min after the injection of systemic morphine (20 mg/kg); ‡ Value is mean with s.e. mean, measured 20 min after the injection of either naloxone or vehicle (about 65 min after the injection of morphine).

§$P < 0.05$ as compared to postmorphine effect; ** $P < 0.05$ as compared to zero.

To demonstrate that the vasoconstriction associated with the hyperthermia was not simply the result of a direct effect on peripheral vasomotor tone, 3 rats received intrathecal morphine (45 µg) whilst in an ambient temperature of 22°C. After reaching a stable level of hyperthermia (+1.8° ± 0.4°C) a heat lamp was focussed on the body. Care was taken to prevent any direct heating of the tail. Within 30–45 s, before any detectable change in core temperature, tail temperatures were observed to rise 6–10° suggesting that morphine did not produce an obligatory vasoconstriction.

Antagonism of the narcotic effects on temperature by intrathecal naloxone in tolerant and non-tolerant rats

If morphine administered systemically is in fact producing its thermoregulatory effects by a direct action on spinal function, then a local antagonism of this action by the intrathecal injection of naloxone should predictably antagonize the effects of systemically administered morphine. These experiments, the results of which are summarized in Table 1, show that morphine injected intraperitoneally yielded a significant increase in colonic temperature which reached maximum within 45 min after the injection and remained stable for about 3 hours. Vehicle administered intrathecally 45 min after the morphine had no effect on the developed hyperthermia whereas, a similar injection of naloxone (5 µg) administered at this time produced approximately a 60% reduction in the magnitude of the temperature response. As indicated in Table 1 this reduction, though statistically significant, did not reach pre-morphine levels. In four animals, ten times this dose of naloxone (50 µg) failed to produce any greater reduction. Full reversal of the hyperthermic effects of systemic morphine could, however, be achieved by the administration of naloxone (2 mg/kg) systemically.

As hypothermia has been reported following the precipitated withdrawal of morphine from tolerant rats (Martin, Wikler, Eades & Pescor, 1963) and as we have observed precipitated withdrawal following intrathecal naloxone (Yaksh, Kohl & Rudy, 1977) we examined the effects of naloxone in the spinal cord on body temperature of rats chronically treated with and tolerant to morphine. Five rats were injected twice daily for 7 days (06 h 00 min and 18 h 00 min) with morphine (20 mg/kg i.p.). While on the first day, these injections produced a mild hyperthermia (+1.34 ± 0.56°C; mean ± s.e. mean), by the 7th day, such injections produced no change in the animal's baseline temperature (0.36 ± 0.43°C). At 12 h 00 min on the 7th day, rats were placed in the restraining cages and following the establishment of a baseline record, received an intrathecal injection of naloxone (10 µg total dose). Figure 3 shows the rapid fall in body temperature produced in the 5 animals following injection of the antagonist. Tail temperatures, measured in two animals indicated that the injection of naloxone produced a marked vasodilatation (6 and 7.5°C rise in tail temperature) while the recovery to the pre-naloxone levels was associated with a fall in tail temperature during the period of recovery. As in the experiments shown in Figure 2, as body temperature approached the pre-naloxone levels in these tolerant animals, tail temperature fell indicating an active vasoconstriction.

In view of the attenuation of the hyperthermic effects of morphine, we sought to examine whether naloxone alone in the range of doses employed here had any effects on body temperature. Systemic injections of naloxone 2 mg/kg in 5 rats had no systemic effect on temperature for a period of up to 4 h after injection (mean ± s.e. mean of maximum temperature change observed during 4 h = +0.35 ± 0.42°C). Similarly, the injection of naloxone (10 µg) intrathecally had no effect on body temperature as shown in Figure 3.

Discussion

That the action of the morphine injected into the spinal subarachnoid space is limited to this region and does not gain access in significant quantities to supraspinal structures has been examined in detail elsewhere (Yaksh & Rudy, 1976b; 1977a,b). Injections of radiolabelled morphine failed to appear in brain during the period after the intrathecal injection. Distribution of label along the cord following an intrathecal injection in fact revealed that the maximum spread of label did not exceed a distance of approximately 4 cm from the catheter tip. More importantly, the time course of the hyperthermic effect was too rapid to be coincidental with the diffusion of a compound which, due to its low lipophilicity, passes the blood brain barrier slowly. Similarly, examination of the dose-response curves for intrathecal morphine indicates that 45 µg produced about a 2°C rise in body temperature. Injections of that amount of morphine systemically (i.e. about 0.15 mg/kg for a 350 g rat) have consistently failed to produce any reliable change in colonic temperature.

The observed hyperthermia was a specific effect of morphine on opiate receptors. The effect was dose-dependent and treatment with indomethacin failed to have any influence upon the effect of the narcotic; this probably excludes the prostaglandins as having an intermediary role in the hyperthermia produced by intrathecal morphine.

Intrathecally administered naloxone reduced the hyperthermic effects of systemically administered morphine, thus supporting the view that the thermogenic effects are mediated in part by an action on the spinal cord. However, even high doses of intrathecal naloxone failed to antagonize completely the hyperthermia produced by systemic morphine. The fact that a subsequent systemic injection of naloxone could in fact produce such a complete reversal suggests that a portion of the hyperthermia produced by systemically administered morphine might be mediated by a pharmacological action of the narcotic on brain regions not directly accessible to the intrathecally injected naloxone. A likely alternative site of action would be the anterior hypothalamus (see Introduction for references). Similarly, Yaksh & Rudy (1977b) observed that the analgesia produced by high doses of systemic morphine was not antagonized completely by intrathecal naloxone. Such results suggest that alternative brain sites may also subserve the analgesic action of morphine and are in accord with the observation that opiates can exert their analgesic effect by an action on several supraspinal structures (Jacquet & Lajtha, 1974; Sharpe, Garnet & Cicero, 1974; Pert & Yaksh, 1974; Yaksh, Yeung & Rudy, 1976; Takagi, Doi & Akaike, 1976).

We suggest that the common factor in these analgesic and thermogenic effects of intrathecal morphine is the modulation of sensory transmission. Just as the perception of and response to pain is not organized at the spinal level, the change in the thermoregulatory set point observed here does not indicate that the centre for thermoregulation is at the spinal level. An animal's thermoregulatory response is in part dictated by input from the peripheral thermal environment. Thus, cooling of the skin evokes predictable and co-ordinated efforts to gain heat. The same behavioural effects could be achieved if a systematic alteration in the thermal information received by supraspinal regulatory centres through the spinal sensory system, were achieved. We believe that the results of the present experiments and the known action of morphine in the spinal cord (see below) is consistent with such a possibility.

Considerable physiological and anatomical evidence exists to support the proposed existence of an endogenous, opiate-sensitive, modulatory system located within the spinal cord. Physiologically, it has been demonstrated that morphine administered into the spinal animal can in fact reduce the discharge of neurones located in lamina 5 of the dorsal horn (Iwata & Sakai, 1971; Besson, Wyon-Maillard, Benoist, Conseiller & Hamann, 1973; Kitahata, Kosaka, Taub, Bonikos & Hoffert, 1974; Le Bars, Menetrey, Conseiller & Besson, 1975), as well as selectively antagonize activity in the ventrolateral tract of the spinal cord evoked by peripheral stimulation (Grossman & Jurna, 1974; Jurna & Grossman, 1976). This spinal tract carries thermal information (Wünneberg & Brück, 1970). Patients in which ventrolateral cordotomies have been performed commonly report a loss of temperature sensation (White & Sweet, 1969). With regard to the locus of narcotic action, recent autoradiographic studies have demonstrated that stereospecific narcotic binding occurs with the substantia gelatinosa of the spinal cord (LaMotte, Pert & Snyder, 1976; Atweh & Kuhar, 1977). Significantly, this complex, polyneuronal region has been suggested as a probable locus for a physiological system mediating the passage of sensory information (Wall, 1973; Melzack & Wall, 1965). Direct evidence linking the pharmacological action of morphine in the substantia gelatinosa to these effects on activity in lamina 5 of the dorsal horn has recently been presented by Duggan, Hall & Headley (1976). They showed that morphine, applied by iontophoresis into the substantia gelatinosa produced an antagonism of the evoked activity recorded in the underlying lamina 5 cells.

Therefore it is likely that intrathecally administered narcotics act upon a modulatory system located within the dorsal horn, presumably in the regions of the stereospecific binding within the substantia gelatinosa, to modulate the passage of sensory information. The opiate-sensitive system may be primarily associated with the input of small fibre systems such as are thought to transmit thermal and nociceptive information (Bessou & Perl, 1969; Price & Wagman, 1970). Our results may be due to an action on these systems.

The present results do not subtract from the apparent importance of rostral diencephalic elements in regulating body temperature. However, significant thermoregulatory effects may be initiated by drugs acting on neural systems in the spinal cord. Whether such systems play a natural role in thermoregulation remains to be considered. The possibility that drugs may produce their effects on thermoregulation by an action at the spinal level, clearly precludes any simplistic interpretation limited to the hypothalamus.

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(Received February 21, 1977.
Revised March 22, 1977.)

Br. J. Pharmac. (1977), **61**, 97–100

COMPARATIVE INHIBITORY EFFECTS OF 3-QUINUCLIDINYL BENZILATE (QNB) AND ATROPINE ON AMYLASE RELEASE FROM RAT PANCREAS

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1 Secretion of amylase from rat pancreas stimulated by urecholine was studied in relation to its inhibition by atropine and 3-quinuclidinyl benzilate (QNB).

2 Urecholine-induced secretion was completely abolished by atropine at 10^{-6} M and QNB at 10^{-8} M. Significant inhibition could be detected at 10^{-8} M atropine and 10^{-10} M QNB.

3 The secretory response to pancreozymin was not blocked either by atropine or QNB.

4 The inhibitory effect of QNB remained for at least 90 min after its removal from the incubation medium.

5 It is concluded that QNB is 10 times more potent than atropine in inhibiting pancreatic amylase secretion induced by urecholine.

Introduction

Pancreatic enzyme secretion is controlled by gastrointestinal hormones and the neurotransmitter acetylcholine. The nervous control of enzyme release has been emphasized recently by reports showing that atropine can prevent depolarization of the acinar cell (Matthews & Petersen, 1973), cellular cyclic GMP accumulation (Christophe, Frandsen, Conlon, Krishna & Gardner, 1976) and calcium efflux (Matthews, Petersen & Williams, 1973; Heisler, 1974) stimulated by cholinomimetic agents.

In order to evaluate further the importance of the parasympathetic nervous system in the control of pancreatic exocrine physiology, studies were undertaken to investigate the antimuscarinic properties of 3-quinuclidinyl benzilate (QNB). This drug has been previously reported to be a potent central muscarinic antagonist (Albanus, 1970) which also inhibits the acetylcholine-induced contractile response of the guinea-pig ileum (Becker, 1974; Yamamura & Snyder, 1974a). The aim of this work was thus to compare the antimuscarinic effects of QNB with those of atropine on pancreatic amylase secretion stimulated *in vitro* by urecholine and the duodenal hormone pancreozymin.

Methods

Male albino rats of Sprague-Dawley strain were used, weighing 300–350 g; they were maintained on Purina Rat Chow and had free access to water. In order to limit variations in rates of enzyme secretion, the animals were submitted to the following schedule: a 24 h fasting period followed by 15 h of feeding and an additional 24 h fast.

In vitro *incubation and secretion studies*

Rats were killed by decapitation and the pancreas of each removed; fat and excess tissue were trimmed. The tissue was weighed on a Roller-Smith balance and pieces of pancreas (350 mg) were incubated in a tissue culture medium (5 ml) under an atmosphere of 95% O_2 and 5% CO_2 as previously described (Beaudoin, Marois, Dunnigan & Morisset, 1974). A 10 min preincubation period was used to equilibrate the tissue and wash out amylase from damaged cells. The tissue was transferred to a new flask with freshly oxygenated medium containing the various drugs and incubated for a 60 min period. At the end of this incubation period, tissue was homogenized in 5 ml of Krebs Ringer phosphate at pH 7.4 with 2.5 ml of perchloric acid 2.1 N for DNA determination. Amylase activity released into the incubation medium was used to monitor enzyme secretion.

Reversibility of the 3-quinuclidinyl benzilate effect

Reversibility of the QNB effect was studied as follows: after 10 min of preincubation, pancreases were exposed to QNB (10^{-8} M) for 30 min; two successive

15 min wash out periods were then performed in freshly oxygenated medium followed by urecholine (10^{-5} M) stimulation for 60 minutes.

DNA and amylase determinations

DNA was determined according to Volkin & Cohn (1954) with calf thymus DNA used as the standard. Amylase activity was assayed according to Bernfeld (1955) with lintner starch as substrate; a unit of amylase activity is that liberating 1.0 μmol of reducing groups calculated as maltose, per minute at 37°C. Data were analysed by Student's *t* test.

Drugs

Urecholine was purchased from Merck, Sharp and Dohme (Kirkland, Quebec). Pancreozymin was obtained from the Gastrointestinal Hormone Research Laboratories, Karolinska Institute (Stockholm, Sweden). 3-Quinuclidinyl benzilate was obtained from H. Landau (New York, N.Y.) and atropine from Sigma (St. Louis, Missouri).

Results

Amylase release is increased by 4.5 fold over the control in the presence of urecholine 10^{-5} M. Atropine and QNB produced a concentration-dependent inhibition of amylase secretion initiated by 10^{-5} M urecholine. Significant inhibition of urecholine-stimulated amylase secretion could be detected at 10^{-8}M atropine and maximum inhibition was observed at 10^{-6} M. QNB produced a significant inhibition at 10^{-10} M which was maximum at 10^{-8} M (Figure 1, Table 1). From Figure 1, the ID_{50} for QNB was estimated at 4×10^{-10} M and for atropine at 4×10^{-9} M. The ID_{50} represents the concentration of antagonist which inhibits the secretory response to urecholine 10^{-5} M by 50%. QNB is thus 10 times more potent than atropine.

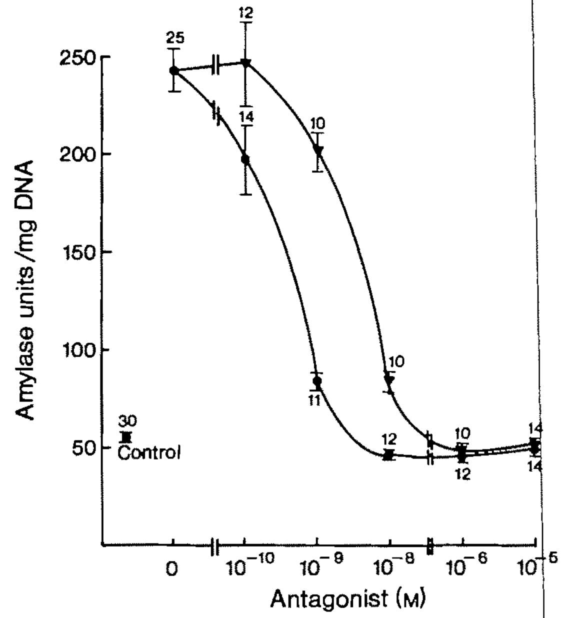

Figure 1 Effects of 3-quinuclidinyl benzilate (QNB) and atropine on urecholine-stimulated amylase release from rat pancreatic tissue. Pancreatic tissue was incubated in tissue culture medium as described in the methods section. For each experiment, urecholine concentration was 10^{-5} M. Each point is the mean value; the figure beside each point indicates the number of tissue pieces in each group Vertical lines show s.e. mean. The controls represent the sum of all controls for each separate experiment. (●) Urecholine + QNB; (▼) urecholine + atropine.

Table 2 shows that the secretory effect of pancreozymin (0.4 IVY unit/ml) was not impaired by QNB 10^{-5} M; this same concentration of the anti-muscarinic agent did not modify basal release of amylase. The inhibitory effect of QNB (10^{-8} M) remained for at least 90 min after its removal from the incubation medium (Table 3).

Table 1 Percentage inhibition of urecholine-stimulated amylase release from rat pancreatic tissue by atropine and 3-quinuclidinyl benzilate (QNB)

	% inhibition			
Concentration (M)	*Atropine*	P *value*	*QNB*	P *value*
10^{-5}	100	<0.001	100	<0.001
10^{-6}	100	<0.001	100	<0.001
10^{-8}	85	<0 001	100	<0.001
10^{-9}	22	>0.05	85	<0.001
10^{-10}	0		24	<0.05

Pancreatic tissue was incubated in tissue culture medium as described in the methods section. For each experiment, the concentration of urecholine was 10^{-5} M. The percentage inhibition was calculated after subtraction of basal amylase release. *P* value was evaluated between means of amylase release from urecholine and those of both antagonists.

Table 2 Effects of 3-quinuclidinyl benzilate (QNB) on basal and pancreozymin stimulated amylase release from rat pancreatic tissue

Groups	*No. of samples*	*Amylase units/ mg DNA*
Control	30	65 5 ± 3 4
Pancreozymin	5	209.7 ± 11.5
Pancreozymin plus QNB	6	212.6 ± 12.6
QNB	9	68.5 ± 6.2

Pancreatic tissue was incubated in tissue culture medium as described in methods. Pancreozymin (GIH) was added at a concentration of 0.4 IVY unit/ml and QNB at a concentration of 10^{-5}M. Results are the mean ± s.e.

Table 3 Irreversible inhibition by 3-quinuclidinyl benzilate (QNB) of urecholine-stimulated amylase release from rat pancreatic tissue

Groups	*No. of samples*	*Amylase units/ mg DNA*
Urecholine	5	432.4 ± 40.9
Urecholine plus QNB	9	79.3 ± 7.1

Pancreatic tissue was incubated in tissue culture medium as described in methods. Urecholine was added at a concentration of 10^{-5} M and QNB at 10^{-8} M. Results are the mean ± s.e.

Discussion

Atropine has been shown to abolish several reactions related to the exocytosis process of pancreatic enzymes stimulated by cholinergic agents. These include depolarization of the acinar cells (Matthews & Petersen, 1973), cyclic GMP accumulation in the cytosol (Christophe *et al.*, 1976), calcium efflux (Matthews *et al.*, 1973; Heisler, 1974) and enzyme release (Morisset & Webster, 1970; Heisler, 1974; Williams, 1975). The present study, using pieces of rat pancreatic tissue, shows that atropine produces a concentration-dependent inhibition of amylase release initiated by urecholine. This inhibition curve (Figure 1) correlates very well with those previously obtained by Christophe *et al.* (1976) on cyclic GMP accumulation and by Gardner, Conlon, Klaeveman, Adams & Ondetti (1975) on calcium efflux.

The pharmacological effects of 3-quinuclidinyl benzilate and its derivatives have been studied in dog, mouse and cat (Albanus, 1970) and in guinea-pig (Yamamura & Snyder, 1974a); these compounds elicited anticholinergic activities i.e. tachycardia, mydriasis, inhibition of salivation and inhibition of acetylcholine-induced contractions of the longitudinal muscle of the guinea-pig ileum. Our data indicate that QNB is 10 times more potent than atropine as an inhibitor of pancreatic enzyme secretion *in vitro*. The observation that QNB does not affect pancreozymin-stimulated amylase release is similar to what was found with atropine (Morisset & Beaudoin, 1977) and shows its antimuscarinic specificity. The irreversible inhibition of amylase release noticed 90 min after QNB removal from the medium demonstrates its long-lasting action; similar results were obtained *in vivo* on salivation and light reflex of the eye in dogs (Albanus, 1970) and on contractions of the guinea-pig ileum (Yamamura & Snyder, 1974a).

Studies are now in progress (Ng, Poirier & Morisset, 1977) to characterize the pancreatic muscarinic cholinoceptor from plasma membranes using [^{3}H]-QNB, as in rat brain (Yamamura & Snyder, 1974b; Snyder, Chang, Kuhar & Yamamura, 1975). Such studies will be helpful to evaluate further the role of the parasympathetic nervous system in the control of pancreatic enzyme secretion.

This work was supported by the NRC of Canada: grants A-6369 and D-63.

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Br. J. Pharmac. (1977), **61**, 101–107

THE CARDIOVASCULAR RESPONSE TO DIMAPRIT, A SELECTIVE HISTAMINE H_2-RECEPTOR AGONIST

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1 The cardiovascular responses to dimaprit (S-(3-dimethylaminopropyl) isothiourea), a selective histamine H_2-receptor agonist have been studied in anaesthetized cats, dogs, rats and rabbits.

2 Dimaprit lowered systemic arterial blood pressure in all species whether given by intravenous injection or by continuous infusion. Intra-arterial injections or infusions caused vasodilatation in the femoral vascular bed.

3 In the cat, continuous intravenous infusions of dimaprit lowered blood pressure without any significant change in heart rate or cardiac output. The fall in blood pressure was thus due to a decrease in peripheral vascular resistance.

4 Dimaprit, given intravenously, increased coronary and gastric blood flows and there were associated marked reductions in vascular resistance. Small reductions in vascular resistance occurred in skeletal muscle, in the intestine and the kidneys.

5 The histamine H_2-receptor antagonists, metiamide and cimetidine, antagonized the depressor and vasodilator responses to dimaprit.

Introduction

The cardiovascular response to histamine has been shown to involve both histamine H_1- and H_2-receptors (e.g. Black, Owen & Parsons, 1975; Tucker, Weir, Reeves & Grover, 1975; Powell & Brody, 1976). The identification of histamine receptors in the cardiovascular system, and elsewhere, has been based predominantly on the use of selective histamine receptor antagonists and confirmed with relatively selective receptor agonists (e.g. Owen, 1975; Powell & Brody, 1976.

Until recently, the most selective histamine H_2-receptor agonist described was 4-methylhistamine (Black, Duncan, Durant, Ganellin & Parsons, 1972) and, although this compound shows marked selectivity for H_2-receptors some residual H_1-receptor activity remains (Durant, Ganellin & Parsons, 1975) and the depressor responses to large doses are due in part to interaction with histamine H_1-receptors (Owen, 1975).

A more selective histamine H_2-receptor agonist, dimaprit, S-(3-dimethylaminopropyl) isothiourea, has recently been described (Parsons, Owen, Durant & Ganellin, 1977). Dimaprit increases the rate of beating of isolated right atria of guinea-pigs, stimulates gastric acid secretion in cats, rats and dogs and elicits depressor and vasodilator responses in cats (Parsons *et al.*, 1977). These responses to dimaprit are due to interaction with H_2-receptors; responses to dimaprit independent of histamine H_2-receptors have not been observed. This paper describes some cardiovascular effects of dimaprit in a number of species.

Methods

Cats

The effects of dimaprit on blood pressure, heart rate and femoral vascular resistance were determined in cats anaesthetized with sodium pentobarbitone (60 mg/kg intraperitoneally). The trachea was cannulated. Blood pressure was measured from the right femoral artery, with a Statham P23A blood pressure transducer, and monitored on a Devices recorder. Heart rate was measured from the blood pressure pulse. Drugs were administered via cannulae in the right femoral vein or right brachial vein. Dose-response curves were constructed for dimaprit by intravenous injection of increasing doses at intervals of 5 min or by continuous intravenous infusion at 0.2 ml/minute.

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The vasodilator effects of dimaprit were determined in the acutely denervated left hind-limb. Systemic blood pressure was measured from a cannula in the right common carotid artery. The left hind-limb was acutely denervated by severing the sciatic nerve bundle in the popliteal cavity. The cat was given heparin 100 iu/kg. The right femoral artery was cannulated and blood pumped through silicone rubber tubing by a Watson-Marlow flow inducer at constant pulsatile flow into the left femoral artery. A small segment of thick rubber tubing was inserted into the external perfusion circuit before the pump for injection of drugs. The perfusion pressure was measured by a blood pressure transducer from a side arm in the perfusion circuit between the pump and the perfused hind-limb. At the start of the experiment the rate of flow was adjusted so that the perfusion pressure was approximately equal to systemic blood pressure and was kept constant at this rate for the duration of the experiment. Dimaprit was administered by intra-arterial injection into the external perfusion circuit before the pump in a volume of 10–20 µl or by continuous intra-arterial infusions at 0.06 ml/minute.

The H_2-receptor agonist activity of dimaprit relative to histamine in causing depressor and vasodilator responses was measured in cats treated with mepyramine, 2.5×10^{-5} mol/kg, to abolish the H_1-receptor responses to histamine.

The effects of infusions of dimaprit on blood pressure, heart rate, cardiac output, stroke volume, total peripheral resistance and regional blood flow were determined in 6 cats anaesthetized by an intraperitoneal injection of urethane, 700 mg/kg, and chloralose, 60 mg/kg. The trachea was cannulated. Blood pressure was measured from the right brachial artery. Heart rate was measured from the blood pressure trace. Cardiac output and regional blood flow were measured using radioactive microspheres (25 µm diameter) by the method described by Johnston & Owen (1977). A catheter was placed in the left ventricle of the heart via the right carotid artery to allow injection of microspheres into the heart. A second catheter was tied into the right femoral artery for withdrawal of blood at a known rate, 5 ml/min, during the injection of microspheres.

Microspheres suspended in 0.5 ml of 0.9% w/v NaCl solution (saline) and labelled with one of two different nucleides were injected, approximately 100,000 microspheres each time. Initially, cardiac output and tissue blood flows were measured under control conditions using microspheres labelled with ^{46}Sc. A second determination of cardiac output and tissue blood flows was made, 30 min after the start of infusions of dimaprit, 5×10^{-7} mol kg^{-1} min^{-1}, with the infusion still running, using microspheres labelled with ^{85}Sr. On completion of the experiments the animals were killed by an overdose of sodium pentobarbitone and the organs of interest were dissected free of surrounding tissue, weighed and transferred to vials to permit measurement of tissue radioactivity in a Nuclear Enterprise 8312 Detector. The channels on the detector were set to provide optimal differential counting conditions for both nucleides. The lungs were counted for radioactivity. The number of microspheres in the lungs did not exceed 5% of the injected total, indicating that very few of the microspheres were not trapped in capillary beds downstream from the site of injection. The even distribution of microspheres was indicated by the close correlation between blood flow to both kidneys. (See Table 1). The microspheres were purchased from the 3M Company.

Dogs

Experiments were carried out in Beagle dogs anaesthetized with intravenous sodium pentobarbitone, 30 mg/kg. Supplementary doses of sodium pentobarbitone were given as required. The dogs were intubated. Blood pressure was measured from the right femoral artery and drugs given via the left femoral vein and the right brachial vein.

Rats

Rats of either sex, 200–250 g, were anaesthetized by an intraperitoneal injection of allobarbitone, 100 mg/kg, plus urethane 400 mg/kg. The trachea was cannulated. Blood pressure was recorded from the right common carotid artery and drugs were administered via cannulae in either femoral vein.

Rabbits

Rabbits of either sex were anaesthetized by intraperitoneal injection of a mixture of urethane, 700 mg/kg plus sodium pentobarbitone, 40 mg/kg. The trachea was cannulated. Blood pressure was measured from one femoral artery and drugs given via the opposite femoral vein.

Drugs

Dimaprit, metiamide, cimetidine, diphenylpyraline hydrochloride, chlorpheniramine maleate and mepyramine maleate were used. Solutions of metiamide and cimetidine were prepared by dissolving the base in a small quantity of 0.1 N HCl, neutralizing by addition of 0.1 N NaOH and made up to volume with saline. The other compounds were prepared in saline.

Results

Intravenous administration

Dimaprit decreased blood pressure in cats, dogs, rats and rabbits. The quantitative nature of this response has been studied in cats.

Intravenous injections of dimaprit to cats caused dose-dependent falls in blood pressure over the dose-range 1×10^{-8} to 1×10^{-6}6 mol/kg (Figure 1). This hypotension was associated with a large increase in pulse pressure and usually persisted longer than the hypotension induced by histamine. Unlike the depressor responses to histamine, which are usually followed by a secondary pressor response, dimaprit did not cause any secondary increase in blood pressure after the initial depressor response (Figure 1). The secondary pressor responses to histamine were usually associated with increases in heart rate; dimaprit did not cause any consistent changes in heart rate.

The depressor responses to dimaprit were unaltered by the administration of the histamine H_1-receptor antagonists, mepyramine, up to 2.5×10^{-5} mol/kg, chlorpheniramine, up to 3.6×10^{-5} mol/kg or diphenylpyraline, up to 3.1×10^{-5} mol/kg. Histamine H_2-receptor blockade with either metiamide or cimetidine caused dose-dependent parallel displacement of the dimaprit dose-response curves to the right (Figure 2). Infusion of metiamide, 4×10^{-7} mol kg^{-1} min^{-1}, caused displacement of the dimaprit dose-response curve with a dose-ratio of 5.7 (3.0–9.8, 95% confidence limits); cimetidine, 4×10^{-7} mol $kg^{-1}min^{-1}$, caused displacement of the dimaprit curve with a dose-ratio of 7.9 (3.4–18.5).

Increasing the infusion rate of both metiamide and cimetidine to 2×10^{-6} mol $kg^{-1} min^{-1}$ increased the dose-ratio to 55.7 (32.3–96.1) for metiamide and to 119.0 (51.1–278) for cimetidine. Even after the administration of either of the H_2-receptor antagonists, when large doses of dimaprit were needed to elicit depressor responses, no further displacement of the dimaprit dose-response curve occurred after histamine H_1-receptor blockade. Relative to histamine, dimaprit has 18.2% (9.9–33.6) the activity to cause depressor responses.

In cats, infusions of dimaprit caused sustained falls in blood pressure. The threshold infusion rate was approximately 1×10^{-7} mol $kg^{-1} min^{-1}$ when diastolic pressure fell, usually with little or no change in systolic pressure. Larger doses of dimaprit, up to 1×10^{-6} mol $kg^{-1} min^{-1}$ reduced both systolic and diastolic

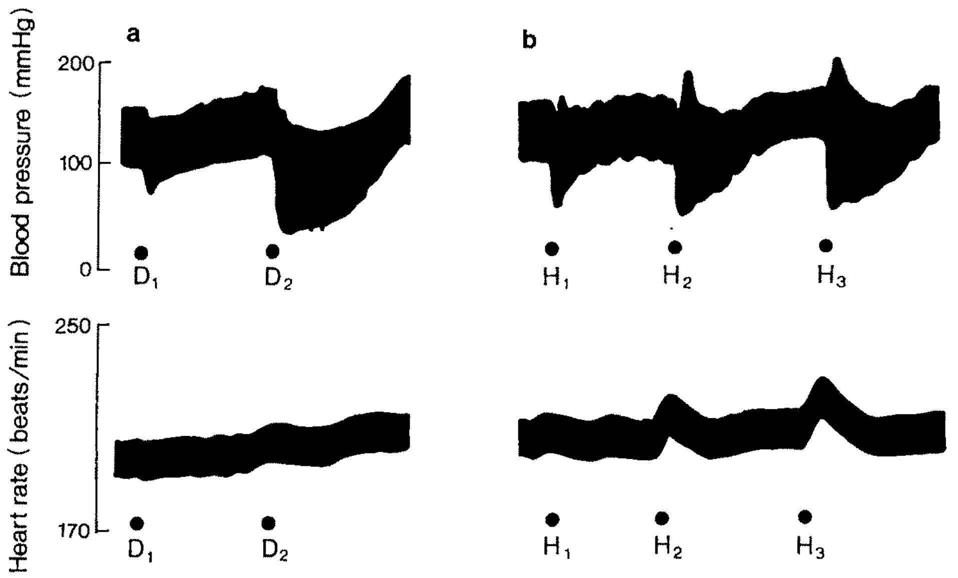

Figure 1 Blood pressure and heart rate responses in an anaesthetized cat. The left hand panels show reductions in blood pressure after intravenous injections of dimaprit, 1×10^{-7} mol/kg at D_1 and 1×10^{-6} mol/kg at D_2. These doses of dimaprit had no effect on heart rate The right hand panels show comparable falls in blood pressure (and increases in heart rate) after injection of histamine, 1×10^{-9} mol/kg at H_1, 1×10^{-8} mol/kg at H_2 and 1×10^{-7} mol/kg at H_3. Unlike histamine, dimaprit does not cause any secondary increase in systolic blood pressure.

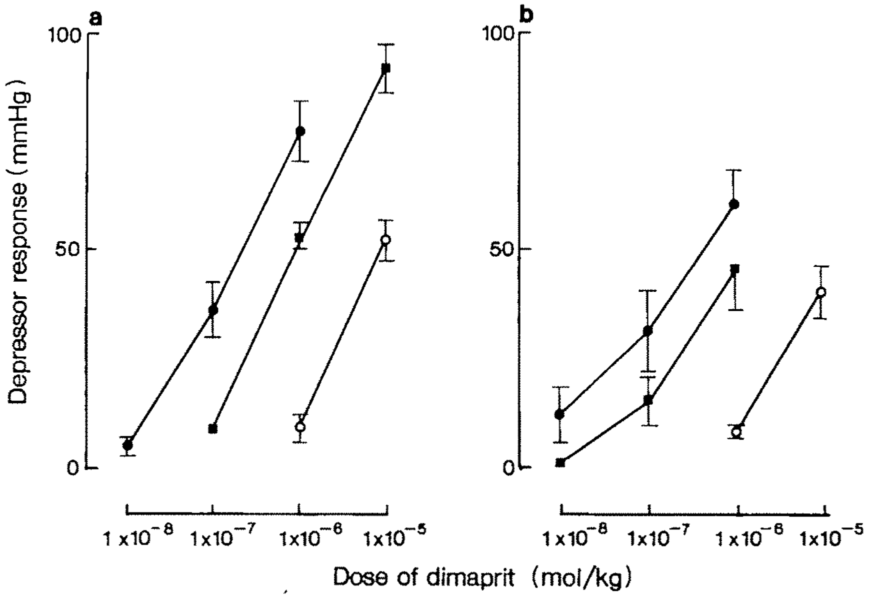

Figure 2 Blood pressure responses to dimaprit in anaesthetized cats. (a) Shows the effect of metiamide and (b) the effect of cimetidine on the dimaprit dose-response curve. Responses in untreated cats (●), during treatment with metiamide or cimetidine, 4×10^{-7} mol kg^{-1} min^{-1} (■), and during treatment with metiamide or cimetidine 2×10^{-6} mol kg^{-1} min^{-1} (○). Each point is mean from 4 cats. Vertical lines show s.e. means.

pressures although the decrease in diastolic pressure always exceeded that of the systolic pressure and pulse pressure was thus increased. Administration of either metiamide or cimetidine during the continuous intravenous infusion of dimaprit (5×10^{-7} mol kg^{-1} min^{-1}) caused a dose-dependent reversal of the depressor response to dimaprit. Both antagonists were roughly equipotent. Complete antagonism of the response to dimaprit occurred after about 1×10^{-5} mol/kg of either antagonist. The dose required for 50% blockade was about 1×10^{-6} mol/kg.

In rats, intravenous injections of dimaprit, 1×10^{-6} mol/kg and 1×10^{-5} mol/kg caused dose-dependent reductions in blood pressure. The responses to both these doses were abolished during infusions of metiamide, 2×10^{-6} mol kg^{-1} min^{-1} although increasing the dose of dimaprit to 1×10^{-4} mol/kg restored depressor responses. In rats, intravenous infusions of dimaprit in excess of 1×10^{-7} mol kg^{-1} min^{-1} caused sustained depressor responses.

In dogs, intravenous infusions of dimaprit, 1×10^{-7} mol kg^{-1} min^{-1} caused a small fall in diastolic blood pressure without any change in systolic pressure. Increasing the dose to 1×10^{-6} mol kg^{-1} min^{-1} caused a large fall in diastolic pressure with a smaller fall in systolic pressure. Administration of cimetidine, 1×10^{-5} mol/kg during continuous infusion of dimaprit abolished the response to dimaprit.

In rabbits, unlike other species studied, histamine causes a biphasic change in blood pressure characterized by an initial increase in pressure, due to histamine H_1-receptor interaction followed by a histamine H_2-receptor depressor response (Parsons & Owen, 1973). Dimaprit, 1×10^{-6} mol/kg, caused a fall in blood pressure with no initial pressor phase. Administration of metiamide 2×10^{-6} mol kg^{-1} min^{-1} abolished this response to dimaprit.

Intravenous infusions of dimaprit, 5×10^{-7} mol kg^{-1} min^{-1} in cats caused a significant fall in blood pressure, mean blood pressure fell from 129 ± 8 mmHg before the infusion to 77 ± 6 mmHg during the infusion ($n = 6$). The fall in blood pressure during the infusion of dimaprit was due totally to a fall in total peripheral resistance; resistance was 0.65 ± 0.04 mmHg ml^{-1} min^{-1} before dimaprit and 0.38 ± 0.03 mmHg ml^{-1} min^{-1} during the infusion. Dimaprit did not significantly change heart rate (240 ± 16 beats/min to 242 ± 14 beats/min), cardiac output (470 ± 42 ml/min to 476 ± 38 ml/min), or stroke volume (1.98 ± 0.17 ml to 1.8 ± 0.25 ml).

Significant increases in blood flow occurred in the heart and stomach; there were no significant changes in blood flow to the intestine, to the muscles of the calf or to skin sample. Significant reductions in blood flow occurred in the brain, liver, kidneys and adrenal glands. These changes in regional blood flow occurred during systemic hypotension; calculation of vascular resistance indicated marked reductions in the

Table 1 The effect of infusion of dimaprit, 5×10^{-7} mol kg^{-1} min^{-1} on regional blood flow and vascular resistance

Region	*Blood flow (ml/min)* *Before infusion*	*Blood flow (ml/min)* *During infusion*	*Vascular resistance (mmHg ml^{-1} min)* *Before infusion*	*Vascular resistance (mmHg ml^{-1} min)* *During infusion*
Brain	20.11 ± 0.88	10.5 ± 0.58*	6.47 ± 0.51	7.37 ± 0.55
Right kidney	33.42 ± 4.40	26.48 ± 3.38*	4.08 ± 0.46	3.05 ± 0 34*
Left kidney	33.40 ± 3.71	26.07 ± 3.18*	4.05 ± 0.45	3.10 ± 0.35*
Heart	26.57 ± 1.78	57.37 ± 5.05*	4.89 ± 0.23	1 38 ± 0.15*
Stomach	8.45 ± 2.22	34.13 ± 6.87*	24 44 ± 8 74	2.54 ± 0.32*
Small intestine	44.97 ± 5.78	53.32 ± 5.57	3.01 ± 0.28	1.74 ± 0 52*
Large intestine	15.28 ± 1.99	19.27 ± 2.75	9.20 ± 1.50	4.45 ± 0.84*
Liver	97.25 ± 8.37	43.18 ± 7 83*	1.36 ± 0.12	2 05 ± 0.33
Adrenal glands	2.45 ± 0.35	1.48 ± 0.23*	57.28 ± 8.17	55.66 ± 6.51
Calf muscle	3.30 ± 0.57	5.37 ± 1.16	43.49 ± 5.71	17.78 ± 3.67*
Skin (sample only)	4.05 ± 0.71	3.28 ± 1.34	25.58 ± 5.23	35.19 ± 6.58

* Indicates values during dimaprit infusion significantly different from pre-infusion values (paired *t* test) $P < 0.05$.

stomach, heart, calf muscle, intestine and kidneys; there were no changes in resistance in the brain, liver or skin. The values for regional blood flow and vascular resistance before and during the dimaprit infusions are shown in Table 1.

Intra-arterial administration

Dimaprit caused dose-related dilatation in the acutely denervated femoral vascular bed in cats over the dose-range 1×10^{-9} to 1×10^{-7} mol/kg. Administration of

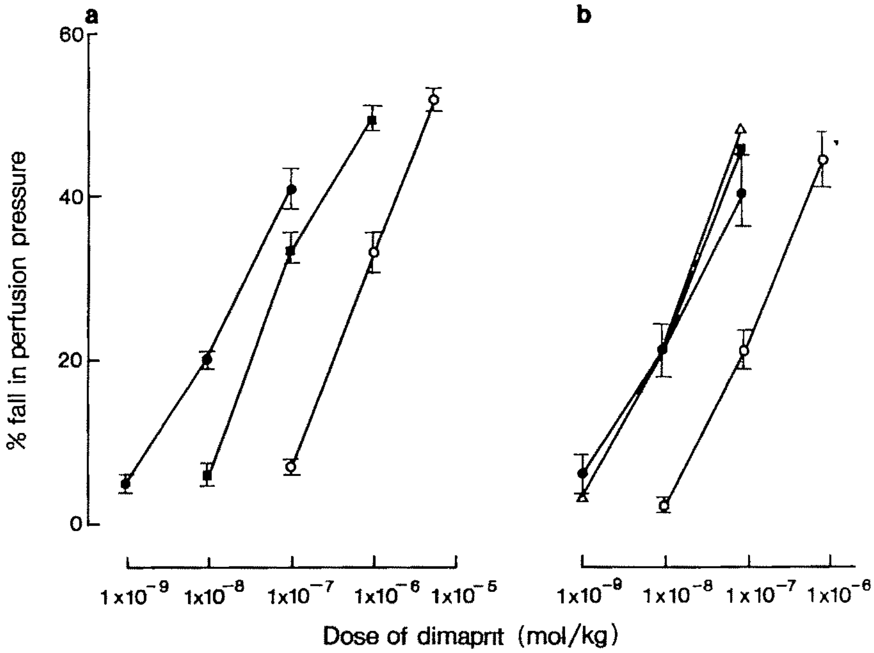

Figure 3 Vasodilatation induced by dimaprit in the femoral vascular bed in anaesthetized cats. (a) Shows displacement of the dimaprit dose-response curve in untreated cats (●) by administration of metiamide, 4×10^{-7} mol kg^{-1} min^{-1} (■), and 2×10^{-6} mol kg^{-1} min^{-1} (○). (b) Shows the failure of mepyramine, 2.5×10^{-6} mol kg (■) or 2.5×10^{-5} mol kg (△) to modify the responses to dimaprit (●). Subsequent administration of metiamide, 4×10^{-7} mol kg^{-1} min^{-1} (○), displaced the dose-response curve to the right.

metiamide caused dose-dependent displacement of the dose-response curve to the right with dose-ratios of 2.74 (1.85–4.05) and 23.2 (15.7–34.4) at 4×10^{-7} mol $kg^{-1} min^{-1}$ and 2×10^{-6} mol $kg^{-1} min^{-1}$ respectively. Subsequent administration of mepyramine 2.5×10^{-6} mol/kg had no further effect on the dimaprit dose-response curve (Figure 3a). Administration of mepyramine alone, up to 2.5×10^{-5} mol/kg, had no effect on the dimaprit dose-response curve (Figure 3b). Dimaprit has 18.6% (16.0–24.0) the vasodilator activity of histamine in the mepyramine-treated cat.

Continuous intra-arterial infusions of dimaprit caused vasodilator responses which persisted throughout the infusion. In 6 cats, an infusion rate of 1×10^{-9} mol $kg^{-1} min^{-1}$ reduced resistance by 8.0 ± 2.3%, 1×10^{-8} mol $kg^{-1} min^{-1}$ reduced resistance by 42.0 ± 4.9%, 1×10^{-7} mol $kg^{-1} min^{-1}$ reduced resistance by 66.0 ± 3.9% and 1×10^{-6} mol $kg^{-1} min^{-1}$ reduced resistance by 64 ± 4.9%.

Discussion

In the present study cardiovascular responses to dimaprit have been shown to involve only H_2-receptors. The responses could be abolished by either of the H_2-receptor antagonists, metiamide or cimetidine and quantitative assessment of this interaction of metiamide or cimetidine indicated that marked and parallel displacements of the dose-response curves to dimaprit occurred. From the use of dose-ratios, it would appear that there is little difference in potency between metiamide and cimetidine. In addition, the dose of either antagonist required to reverse the depressor response to dimaprit was approximately the same as that required to inhibit either histamine or dimaprit-induced gastric acid secretion, Thus the effective dose for 50% inhibition of the dimaprit depressor response was approximately 1×10^{-6} mol/kg. This value is similar to the ED_{50} for metiamide against dimaprit-induced gastric acid secretion in rats (Parsons *et al.*, 1977).

The potency of dimaprit as an H_2-agonist in the cat, relative to histamine, was similar both for depressor and vasodilator responses and similar to their relative potencies in causing gastric acid secretion in the rat and for inhibiting electrically induced contractions of the rat uterus (Parsons *et al.*, 1977).

Like histamine, dimaprit lowered systemic arterial blood pressure when given intravenously. The response differed from that to histamine in that it was more persistent and was never followed by a secondary rise in blood pressure, an effect first observed by Burn & Dale (1926) and due to an H_1-receptor mediated release of catecholamines from the adrenal medulla (Emmelin & Muren, 1949; Black *et al.*, 1975). The greater persistence of the depressor responses to dimaprit almost certainly reflects the absence of catecholamine release opposing the direct depressor effects, although the relative rates of metabolism of dimaprit and histamine may also be important.

Dimaprit usually did not change heart rate despite causing marked systemic hypotension. This failure to elicit reflex tachycardia is consistent with our experience using other vasodilator compounds such as hydralazine (Fielden, Owen & Taylor, 1974) and may, in part, be due to the effect of anaesthesia (Kirchheim, 1976). Although H_2-receptor agonists have been shown to increase sinus rate in isolated cardiac tissue (Black *et al.*, 1972; Broadley, 1975; Levi, Capurro & Lee, 1975), tachycardia in response to H_2-receptor agonists *in vivo* has not been demonstrated. Tachycardia induced by histamine in anaesthetized cats is due, in part, to release of catecholamines from chromaffin tissue (Owen, unpublished) and this is a histamine H_1-receptor phenomenon (Emmelin & Muren, 1949). Dimaprit would not be expected to release catecholamines from chromaffin tissue.

The pattern of depressor responses after dimaprit administration is consistent with results obtained with histamine in mepyramine-treated animals (Black *et al.*, 1975; Flynn & Owen, 1975; Owen 1975; Johnston & Owen, 1977). The response to H_2-receptor agonists consists of dilatation of resistance vessels leading to a decreased vascular resistance. The major sites of dilatation during systemic administration are the coronary, splanchnic and skeletal muscle vasculature. The changes in blood flow in other vascular beds mainly reflect changes in arterial perfusion pressure more than local vascular responses.

In conclusion, this paper characterizes some effects of dimaprit on the cardiovascular system. Dimaprit is likely to prove more valuable than 4-methylhistamine as a tool for examination of histamine H_2-receptor characteristics, because unlike 4-methylhistamine it has no detectable H_1-receptor agonist activity (Parsons *et al.*, 1977).

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(Received February 28, 1977.
Revised March 21, 1977.)

Br. J. Pharmac. (1977), **61**, 109–110

EFFECTS OF *N*-BENZYL-L-VALINE ON CONTRACTIONS OF GUINEA-PIG MYOMETRIUM *in vitro*

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A claim that *N*-benzylvaline is an exceedingly potent stimulant of guinea-pig myometrium has not been confirmed, but less potent stimulant or inhibitory effects were seen in a few experiments.

Introduction Koroza, Kudrin, Kost & Sagitullin (1969) stated that certain *N*-substituted amino acids markedly enhanced the contractions of the guinea-pig uterus, when applied in exceedingly low concentrations for periods of 10–15 minutes. The most potent one was stated to be *N*-benzylvaline (Na salt), which was claimed to intensify contractions at concentrations of the order of 10^{-19} M, or to cause spasm at 10^{-13} M. Since a number of common amino acids at much higher concentrations have recently been shown to stimulate the uterus (Bedwani, Ishizawa, Pickles & Suwankrughasn, 1977), and as there are a few simple *N*-substituted amino acids of pharmacological or physiological interest, it seemed worthwhile to see if the claim by Koroza *et al.* (1969) could be substantiated.

Methods *N*-benzyl-L-valine (NBV) with the addition of the equivalent amount of $NaHCO_3$ was dissolved in Krebs-Henseleit solution to give 10 mM stock solutions, which were kept at 4°C until they were diluted for use. Guinea-pig uteri were suspended in Krebs-Henseleit solution as in the experiments of Bedwani *et al.* (1977), except that animals in oestrus were not avoided; 2 of the 17 animals used appeared to be in this phase. For short-term applications, NBV was applied by injection into the organ bath. When longer-term application was needed, as in the experiment shown in Figure 1a, the NBV was added in the desired final concentration to the reservoir of Krebs-Henseleit solution in a duplicate supply system, so that steady slow flows of medium either with or without NBV could be alternated at will while all other

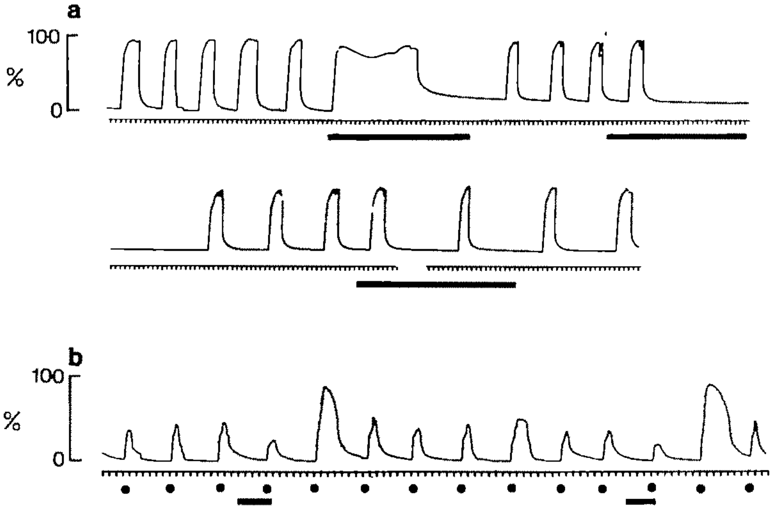

Figure 1 (a) Effects of *N*-benzyl-L-valine (NBV) on spontaneous contractions of a guinea-pig uterus. The medium flowed continuously through the 4 ml organ bath at a rate of 2.8 ml/min; during the periods shown by bars it contained 0.6 µM NBV. The record is continuous. (b) Delayed potentiation of responses to acetylcholine (ACh, 1 µM, 1-min applications at dots) by 4 mM NBV. The potentiation is approximately equivalent to a doubling of the dose of ACh. It was seen in each of 5 similar trials in this experiment, and in a similar one with 4 mM L-valine but in this case without the delay. Time-marker 1 min in both traces. Ordinate scales: % maximum contraction height.

conditions were kept constant. By either means, the bath concentration of NBV was usually within the range 0.5 µM to 1.0 mM.

Results In only 2 of the 17 experiments was a strong effect of the type described by Koroza *et al.* (1969) seen, and this only with higher concentrations (0.6 and 60 µM). One of these is shown in Figure 1a. On repeated application the NBV no longer stimulated, but inhibited the spontaneous contractions. Prolonged inhibition without preceding stimulation was seen in another two experiments. In seven further experiments, NBV was applied for 4–10 min before acetylcholine, prostaglandin E_2 or vasopressin, which were added in its presence, and the responses were compared with those in the absence of NBV. Again, most results were negative but a repeatable delayed potentiation was seen in one experiment, in which an exceptionally high concentration (4 mM) of NBV was used. Part of this experiment is shown in Figure 1b. Inconstant trace responses of various kinds were seen in a few other experiments.

Discussion These results do not support the claim of Koroza *et al.* (1969) for *N*-benzylvaline. However, they do show that this compound, or at least the L enantiomer which we used, has some activity on a minority of uteri. In such instances the effect may be either stimulation or inhibition of the contractions, or one of these effects may lead to the other. The concentration of NBV sometimes producing stimulation is less than that of the common amino acids showing a similar effect (Bedwani *et al.,* 1977), but the potentiation of other spasmogens given by low concentrations of those acids was not given by NBV.

There may be several possible reasons for the difference between the present results and those of Koroza *et al.* (1969) but their account gives no detail of experimental method and speculation would be futile. In the present series, similar preparations from different animals have given widely differing results. Stimulant and inhibitory responses were found with approximately equal frequencies but the two types showed no constant relationship, suggesting two unrelated mechanisms. Their relative slowness (especially for inhibition) may indicate that the actions are indirect; and here it may be noted that Koroza *et al.* (1969) theoretically derived their compounds from two others then in use as monoamine oxidase-inhibitors, which they also said were stimulants of the guinea-pig myometrium. Possibly NBV modulates the spontaneous contractile activity of some uteri by one or more actions on endogenous catecholamine or 5-hydroxytryptamine metabolism.

Professor A.H. Jackson (University College Cardiff) kindly arranged the synthesis of NBV undertaken by Dr Marguerite Games.

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(Received May 30, 1977)

PROCEEDINGS OF THE
British Pharmacological Society

13th–15th July, 1977

UNIVERSITY OF NEWCASTLE UPON TYNE

COMMUNICATIONS

Ontogenesis of the multiple forms of choline acetyltransferase: uptake and acetylation of choline in rat brain

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Rat brain choline acetyltransferase (ChA) exhibits molecular heterogeneity (Malthe-Sørenssen & Fonnum, 1972), but whether the multiple forms are isoenzymes with differing subcellular localizations and functions remains uncertain. There is evidence for the coupling of the high affinity uptake and acetylation of choline in rat brain (Barker, Dowdall & Mittag, 1975; Burgess & Prince, 1977) although the mechanism is unknown. We therefore sought changes in the heterogeneity of ChA and correlated these with changes in uptake and acetylation of choline in developing rat brain.

High speed supernatants and partially purified samples (Malthe-Sørenssen & Fonnum, 1972) from whole brains of 7-day old and adult Wistar rats were analyzed by isoelectric focusing. ChA was assayed radiometrically (Fonnum, 1975). The sodium-dependent high affinity uptake of [^{3}H]-choline (0.5 μM) by small slices of frontal cortex was measured by incubation in Krebs medium (10 min, 37°C). Corrections were made for sodium-independent low affinity uptake. Conversion of labelled choline into acetylcholine was measured (Potter & Murphy, 1967).

Both preparations, from mature and immature brain, possessed the three forms of ChA previously reported (Table 1). The most basic comprised 60% of the total activity recovered after electro-focusing of mature brain. In contrast, in immature brain the most basic form represented only 19% of total recovered activity.

Sodium-dependent high-affinity uptake accounted for approximately 30% of total uptake in brain of 7-day old rats, compared with 55% in the mature brain. 90% of the choline taken up by means of the high affinity mechanism was acetylated in mature brain compared with only 45% in immature brain.

Thus developmental increases in the efficiency of acetylation of choline after high affinity uptake in the

Table 1 Isoelectric focusing of developing rat brain choline acetyltransferase

Preparation of choline acetyltransferase	*Distribution of choline acetyltransferase activity* (%) pH 7.1–7 6	 pH 7.6–8.0	 pH 8.0–8.7
Adult brain (A)	14.38±4.3	28.30±6.7	52.85±5.4
Adult brain (B)	10.53±3.3	17 48±3 6	64.14±7.2
Immature brain (A)	31.18±3.7	43.81±5.9	18.97±2.4
Immature brain (B)	25.20±2.4	29.29±6.2	19.33±0.4

The enzyme preparations were either: (A) high speed supernatant of brain homogenate, or (B) partially purified as described by Malthe-Sørenssen & Fonnum (1972) Isoelectric focusing was carried out in 110 ml LKB columns (4°C), pH 6–9.5, 400 V for 46 hours. The distribution of ChA activity in the different pH ranges is expressed as a % of the total activity recovered after focusing. Residual activity was largely recovered in pH 6.5–7.1 range. Results are means ± s.e. mean of four focusing experiments in each case.

rat, may be related to the appearance of increased quantities of a basic isoenzyme of ChA. A molecular basis for the efficient coupling of sodium-dependent high affinity uptake and ChA will be discussed in light of these results.

Trust for financial support.

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Activation of high affinity choline uptake in sympathetic ganglia by potassium depolarization

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There is evidence to suggest that high affinity uptake processes for choline are associated with cholinergic nerve terminals. Sympathetic ganglia accumulate choline by both high and low affinity uptake processes but we found that denervation of ganglia 10 to 14 days before their removal had no effect on the high affinity choline uptake process (Bowery & Neal, 1975). In the present study we have examined the effect of potassium depolarization on choline transport in ganglia and have found an association between cholinergic nerve terminals and a high affinity choline uptake process.

Isolated rat superior cervical ganglia were desheathed and given a preliminary incubation at 37°C for 30 min in Krebs bicarbonate Ringer. Then $[^{3}\text{H}]$-choline (1 μ Ci/ml) was added to give a final concentration of 0.1 μM or 100 μM and the incubations were continued for 10 minutes. Finally, the ganglia were dissolved in Soluene (Packard) and the radioactivity was measured by liquid scintillation counting. When ganglia were depolarized, potassium chloride (40 mM) was included in the medium both during the preliminary incubation and incubation period. The results are summarized in Table 1.

Potassium depolarization increased $[^{3}\text{H}]$-choline uptake by the high affinity uptake process but decreased transport by the low affinity process. Chronic denervation, absence of sodium or calcium ions in the medium, and increased magnesium ion concentration (20 mM), not only abolished the potassium induced activation of the high affinity choline uptake process, but changed the response to inhibition.

One explanation for these results is that potassium depolarization causes a calcium dependent release of

Table 1 Effect of potassium depolarization on $[^3H]$-choline uptake by rat sympathetic ganglia

Composition of medium for preliminary incubation and incubation	*Choline uptake*			
	'High affinity uptake'		*'Low affinity uptake'*	
	T/M	*% control*	*T/M*	*% control*
Control (Krebs bicarbonate Ringer)	6.9 ±0.4	100	2.68±0.14	100
KCl (40 mM)	9.9* ±0.8	142*	2.12±0.10*	73*
KCl, Ca free medium	5.1* ±0.5	68*		
KCl + $MgSO_4$ (20 mM)	5.2* ±0.2	64*		
KCl, Na free medium	3.5* ±0.3	51*		
KCl, denervated ganglia	4.5* ±0.3	54*		
Denervated ganglia (controls)	6.0 ±0.3	112	2.70±0.27	108

The high and low affinity uptake of choline was estimated by incubating ganglia in a low (0.1 µM) or high (100 µM) concentration of $[^3H]$-choline respectively. The results are expressed as both the tissue : medium ratio (T/M = $dpm.g^{-1}$ wet weight/$dpm.ml^{-1}$) and as the percentage of uptake in control ganglia not exposed to KCl (% control) The 'T/M column' does not necessarily correspond exactly with the '% control column' since only the latter results are calculated from paired controls. The results (T/M) are the mean ± s.e. mean of 6 to 18 determinations.

* Significantly different from controls ($P<0$ 0.5 and >0.001).

ACh from nerve terminals, which then results in activation of a high affinity, sodium dependent, uptake process for choline.

A.J. Higgins is an MRC student.

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Differentiation of neurogenic inhibition from ATP-responses in guinea-pig taenia caeci

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Twin 3–4 cm preparations were suspended in Krebs-Henseleit (1.5 mM Mg^{++}; 30°C, to reduce spontaneous tone-fluctuations) and contracted by carbachol, 27–164 nM. ATP-tachyphylactic muscles were discarded. ATP-relaxations persisted after phenoxybenzamine (2.3 µM) and pindolol (4–40 µM) but were reversibly antagonized by phentolamine, (2.6–13 µM) acting by an unknown mechanism not involving classical adrenoceptors of this muscle.

Neurogenic inhibition. To eliminate the participation of adrenergic fibres and to single out non-adrenergic inhibitory transmission, unatropinized preparations were rendered insensitive to the relaxing effect of phenylephrine, isoprenaline or noradrenaline by combined α- + β-adrenoceptor block following phenoxybenzamine-treatment (2.3 µM; 15 min) and the introduction of pindolol (40.3 µM). Tetrodotoxin-susceptible inhibitions induced by single 0.1–0.5 ms pulses were compared with relaxations induced by low ATP-concentrations (0.14–1.44 µM for 30 s). In all of 11 experiments, phentolamine (13 µM) greatly reduced ATP-relaxations but left neurogenic inhibitions usually unaltered (Figure 1), or potentiated or slightly reduced.

These results confirm in a different way that illustrated in a 'short report' by Rikimaru, Fukushi & Suzuki (1971), who used 10 times more phentolamine (135 µM); and atropine but no phenoxybenzamine or pindolol. Satchell, Burnstock & Dann (1973) (using guanethidine and hyoscine) failed to confirm this in 3 of 5 experiments with phentolamine (45 µM) and in order to achieve ATP-block raised phentolamine concentration to 180 µM, which then depressed transmission as well. But, under the present conditions, phentolamine in the much lower concentration of 13 µM has regularly differentiated neurogenic from ATP-responses. This, and the frequent occurrence of marked tachyphylaxis to ATP suggest that the non-adrenergic inhibitory transmission is not purinergic and may be akin to autonomic inhibitory transmission elsewhere, e.g. retractor penis, where ATP is excludable because it contracts whereas

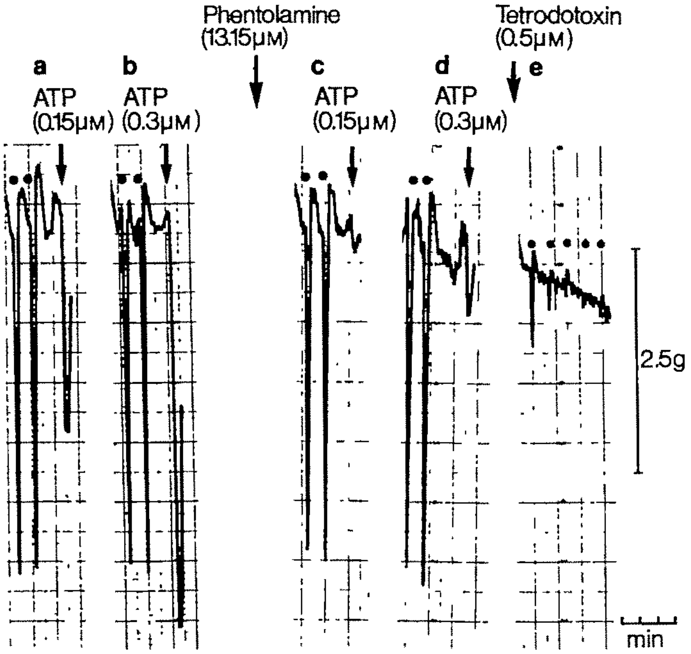

Figure 1 Unatropinized guinea-pig taenia caeci, 30°C; isometric recording. α- and β-adrenoceptors were blocked *ab initio* by phenoxybenzamine (2.3 μM given for 15 min, previously) and pindolol (40.3 μM) in the reservoir. Tone was produced by carbachol (131 nM) renewed before each panel. Panels a–d each show, first, two consecutive inhibitory responses to transmural stimulation with single 0.5 ms pulses of constant voltage, delivered at 1 min intervals, followed, at the arrows, by ATP given for 30 s; the pen recorder was lifted off the paper when ATP was washed out. Phentolamine, (13.15 μM) was introduced 0.5 h before panel c. Panel e shows the extinction of responses to transmural stimulation by tetrodotoxin (0.5 μM). Stimulus deliveries indicated by the dots.

the sacral nerves relax. This effect of phentolamine (13 μmol) was absent in guinea pig vasa or detruser strips (ATP-contractions not antagonized).

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Pharmacology of electrically evoked contractions of human bladder

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Isolated strips of detrusor muscle from rats, guinea-pigs, rabbits and from other species have been used previously to study the nature of motor transmission in the bladder (Ursillo & Clarke, 1956; Hukovic, Rand & Vanov, 1965; Ambache & Zar, 1970). However, no such comparable investigation has been carried out on human bladder.

A total of 14 experiments were carried out on isolated strips (5 x 0.3 cm) of human bladder, obtained from patients undergoing bladder surgery. The preparations were suspended in 10 ml organ baths between parallel platinum electrodes in Krebs-Henseleit solution at 37°C and contractions were recorded isometrically. For electrical field stimulation, trains of 5–20 pulses (0.5 ms duration, 10 Hz, 12 V) were delivered at 60–90 s intervals. Electrical stimulation resulted in contractions which were abolished by tetrodotoxin (0.2 µg/ml) and which were therefore neurogenic. The electrically induced contraction (EIC) was unaffected by hexamethonium, (0.1 mM) suggesting its post-ganglionic origin. EICs were greatly augmented by eserine (3–6 µM) and were partially antagonized by atropine (0.1 µM); no further antagonism was noticed on increasing the atropine concentration to 1–5 µM. The atropine-resistant component of EIC was not potentiated by eserine.

Noradrenaline (and isoprenaline), 0.01–1 µM inhibited EIC drastically; in contrast, acetylcholine-induced contraction was reduced only slightly or not at all by noradrenaline. The inhibition of EIC by noradrenaline remained unaffected by phentolamine (5 µM) but was antagonized to a variable extent by propranolol (1–5 µM).

Phenylephrine and clinidine (0.01–1 µM), either did not affect the EIC or potentiated it; the potentiation was usually seen with the higher concentrations of these drugs.

EIC, but not the acetylcholine evoked contraction, was potentiated by remarkably low concentrations of 5-hydroxytryptamine (0.1– nM). The potentiating effect of 5-hydroxytryptamine remained unaffected by methysergide (0.5 µM) and by morphine (1 µM). Atropinization did not prevent the potentiating effect of 5-HT on EIC.

The foregoing results provide evidence for the view that

(1) acetylcholine is the predominant, but not the sole, motor transmitter in human bladder. This conclusion contrasts with the findings in isolated strips of guinea-pig, rabbit and cat bladder, when the motor transmission was found to be predominantly non-cholinergic (Ambache & Zar, 1970) and is probably a reflection of species variability.

(2) biogenic amines, noradrenaline and 5-hydroxytryptamine possess the ability to play significant but opposite roles in modulating the motor transmission to the detrusor muscle largely through presynaptic mechanisms.

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Temperature-dependent effects of autonomic drugs on the response of the guinea-pig isolated bladder to parasympathetic nerve stimulation

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Contractions of the guinea-pig isolated bladder to pelvic nerve stimulation are only partially blocked by hyoscine or ganglion blocking agents (Weetman, 1972; Weetman & Turner, 1973). Whilst continuing this investigation, an experiment was performed at room temperature because a bath heater was inadvertently not switched on. Hyoscine produced no blockade in this case, so the present experiments were performed.

Bladders were taken from female guinea-pigs (250–400 g) and divided longitudinally to provide paired preparations: one, the control, was used at 33°C, the other at a different temperature. Preparations were arranged in 100 ml isolated organ baths filled with McEwen's solution which was gassed

Table 1 Effects of drugs on paired hemibladder preparations at different temperatures

Drug	*Concentration (μM)*	*n*	*Temperature °C (s.e. mean)*	*Response % control (s.e. mean)*	*P*
Hyoscine	0.3	7	33.7 (0.4)	67 (5)	
			21.4 (0.8)	92 (5)	<0.01
Hyoscine	0.3	4	33.1 (0.6)	56 (12)	
			38.5 (0.5)	47 (12)	>0.05
Hexamethonium	300	4	33.5 (0.5)	45 (9)	
			21.6 (0.6)	85 (5)	<0.01
Eserine	8	6	33.1 (0.4)	162 (17)	
			23.1 (0.5)	120 (6)	<0.01

The pelvic nerves were stimulated maximally with 0.5 ms duration pulses at 8 Hz for 7 s every 2 minutes. Drug-induced changes in the response of the tissues were measured when they became maximal.

with 5% CO_2: 95% O_2. The pelvic nerve was stimulated with bipolar platinum electrodes at 8 Hz for 7 s every 2 min, and contractions of the longitudinal muscle were recorded on a Grass RPS 7C8A polygraph via Grass FTO3C force-displacement transducers.

Hyoscine (0.3 μM) produced a maximum blockade of the response to nerve stimulation that was greatest at 38°C, and least at room temperature (21°C, Table 1). Doubling the stimulation period to 14 s or increasing the concentration of hyoscine to 3 μM did not increase the magnitude of the blockade ($n=3$). In contrast with this, hyoscine (0.3 μM) did block the effects of exogenous acetylcholine at room temperature ($n=4$). Hexamethonium (0.3 mM) was also ineffective against the response to nerve stimulation at room temperature, but not at 33°C. Eserine (8 μM) increased the responses to nerve stimulation more at 33°C than at room temperature (Table 1). The response to electrical stimulation at 21°C was probably due to excitation of nervous elements because tetrodotoxin (0.3 μM) abolished the contractions ($n=2$).

These results indicate that the parasympathetic innervation to the bladder exhibits the pharmacological characteristics of a mixed nerve, the cholinergic component of which is only effective above room temperature. At room temperature the innervation is almost entirely non-cholinergic.

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Evidence for two distinct types of postsynaptic α-adrenoceptor

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Prazosin lowers blood pressure in animals (Constantine, McShane, Scriabine & Hess, 1973) and man (Cohen, 1970). Its primary mode of action is thought to be blockade of vascular α-adrenoceptors (Wood, Phelan & Simpson, 1975; Cavero, 1976). Moulds & Jauernig (1977) showed that prazosin antagonized the contractile effect of noradrenaline in isolated spiral strips of human visceral arteries but, in contrast to phentolamine, was inactive against noradrenaline on peripheral arteries. We have investigated the α-adrenoceptor blocking action of prazosin in more detail by determining its ability to antagonize responses to exogenous phenylephrine and noradrenaline and to sympathetic nerve stimulation *in vivo*. Phentolamine was included in the study for comparison. All drugs were given intravenously.

In pithed rats phentolamine competitively antagonized the vasopressor responses to phenylephrine and noradrenaline. The doses of phentolamine required to produce phenylephrine and noradrenaline dose-ratios of 10 (DR_{10}) were 0.24 and 0.46 mg/kg respectively. Prazosin was a competitive antagonist of phenylephrine; its DR_{10} was

0.0125 mg/kg. In contrast, prazosin had little or no effect on the pressor responses to low doses of noradrenaline (30–300 ng/kg) but reduced those to higher doses (>1 μg/kg). Pretreatment with propranolol or cocaine did not markedly alter the interaction between prazosin and noradrenaline.

The α-adrenoceptor blocking actions of phentolamine and prazosin were also examined in chloralose anaesthetized cats, from which the right adrenal gland had been removed. In these experiments, matched submaximal pressor responses to single doses of phenylephrine and noradrenaline and to right splanchnic nerve stimulation were obtained before and after administration of the antagonist. Phentolamine caused a dose-related reduction in the response to each stimulus; the mean doses of phentolamine which reduced by 50% (ED_{50}) the responses to phenylephrine, noradrenaline and nerve stimulation were 0.20, 0.38 and 1.22 mg/kg respectively. The corresponding ED_{50} values for prazosin were 0.04, 2.69 and >10 mg/kg respectively. Thus prazosin was about 5 times more potent than phentolamine against phenylephrine, but was much less potent against noradrenaline and nerve stimulation.

The results of these experiments suggest that there maybe two types of postsynaptic α-adrenoceptors. Phenylephrine stimulates only one type and prazosin blocks only this type. Low doses of noradrenaline stimulate the prazosin-insensitive type and high doses stimulate both types. Phenotolamine blocks both types of α-adrenoceptor. The general distribution of these two types of receptors is not known but those innervated by the right splanchnic nerve in the cat seem to be predominantly of the prazosin-insensitive type. The prazosin-insensitive receptors may resemble the presynaptic α-adrenoceptors located on the terminals of the adrenergic nerves supplying the rabbit pulmonary artery (Cambridge, Davey & Massingham, 1977) and rat heart (Cavero, Lefèvre & Roach, 1977) at which prazosin is a weak antagonist and phenylephrine is a weak agonist.

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Calcium, cyclic amp and the response of rat descending colon to angiotensin, to prostaglandin E_2 and to potassium chloride

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The increased intracellular calcium for muscle contraction may arise from intracellular or extracellular sources (Van Bremmen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973) perhaps associated with altered cyclic AMP levels (Marshall & Kroeger, 1973). Smooth muscle responses to angiotensin (Khairallah, Vadaparampil & Page, 1965) and prostaglandins (Coceani & Wolfe, 1966) are sensitive to changes in extracellular calcium. The role of calcium and cyclic AMP in the response of rat descending colon to angiotensin, Prostaglandin E_2 and potassium has now been investigated.

Isotonic contractions were recorded of muscle preparations from male Wistar rats suspended in aerated Tyrode solution. Preparations were exposed to a calcium-free Tyrode containing EDTA (0.025 mmol). Half-maximal responses to angiotensin were reduced to $7.8 \pm 1.3\%$ ($n=6$) after 15 min whereas half-maximal responses to prostaglandin E_2 and potassium were reduced to $50.8 \pm 4.9\%$ ($n=6$) and $39.2 \pm 5.6\%$ ($n=6$) respectively. The reduction of angiotensin responses was significantly greater than those of prostaglandin E_2 or potassium ($P<0.001$). SKF525A inhibits calcium influx associated with depolarization (Kalsner, Nickerson & Boyd, 1970). Preparations were exposed to calcium free Tyrode containing 2.6×10^{-5} mol SKF525A for 60 minutes. On reintroduction of calcium the tonic responses to angiotensin and prostaglandin E_2 were reduced and potassium was inactive. Subsequent removal of SKF525A allowed recovery of responses, rapidly for angiotensin and prostaglandin E_2 but slowly for potassium.

Tyrode containing 2,4-dinitrophenol (0.05 mmol) caused little reduction of the phasic responses to angiotensin and prostaglandin E_2 but a progressive reduction of tonic responses to $4.0 \pm 1.9\%$ ($n = 6$) and $3.6 \pm 2.5\%$ ($n = 6$) respectively after 65 minutes. The corresponding potassium response was reduced to $42.0 \pm 6.7\%$ ($n = 6$) ($P < 0.01$).

Angiotensin, prostaglandin E_2 and potassium responses were recorded following a 30 min exposure to Tyrode solution containing imidazole (0.05 mol) or theophylline (0.3 mmol) and plotted as a percentage of the maximum response against log concentration. Imidazole caused a non-specific potentiation of all responses. Theophylline caused a reduction of responses to all agonists but the ED_{50} log concentration shift was significantly greater for angiotensin (0.45 ± 0.05, $n = 6$) and prostaglandin E_2 (0.64 ± 0.16, $n = 6$) than for potassium (0.14 ± 0.03, $n = 6$) ($P < 0.001$). Isoprenaline (5×10^{-9}–1.6×10^{-7} mol) added 30 s before the agonists caused a dose dependent decrease in the responses, the reduction of the potassium responses was significantly less than those of angiotensin or prostaglandin E_2 ($P < 0.001$).

These findings support suggestions that potassium causes depolarization of smooth muscle (Goodman & Weiss, 1971) associated with calcium influx and subsequent intracellular release (Cheng, 1976). Responses to angiotensin and prostaglandin E_2 may consist of two phases, an initial transient associated with depolarization and a sustained tonic response independent of depolarization but dependent upon metabolic energy. This supports other reports of the energy dependence of prostaglandin (Coceani & Wolfe, 1966) and angiotensin (Crocker & Wilson, 1975) responses. This tonic phase of the angiotensin response is more dependent upon extracellular calcium than that of prostaglandin E_2. Increases in cyclic AMP induced by isoprenaline or theophylline had little effect upon potassium responses but markedly reduced angiotensin or prostaglandin E_2 sustained responses which may be related to an effect upon calcium distribution.

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Substrate selective inhibition of monoamine oxidase by mexiletine

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Mexiletine (1-methyl-2-(2,6-xylyloxy)-ethylamine hydrochloride, Kö 1173) is an effective antiarrythmic agent in man and other animals, with a local anaesthetic potency comparable with that of lignocaine, to which it shows a structural similarity (Singh & Vaughan Williams, 1972). However, mexiletine is also an α-substituted monoamine, which suggested that it could inhibit monoamine oxidase (MAO), since many compounds with this structure are known to possess this property (Blaschko, Richter & Schlossmann, 1937; Pugh & Quastel, 1937; Mantle, Tipton & Garrett, 1976).

The hearts, livers and brains of male Wistar rats of 300–350 g body weight, were homogenized in 1 mM potassium phosphate buffer, pH 7.4. The homogenates were centrifuged at low speed to remove unbroken cells and nuclei, and the supernatant fractions used for all experiments. MAO activity was assayed radiochemically with [^{3}H]-tyramine, [^{3}H]-5-HT, [^{14}C]-β-phenylethylamine and [^{14}C]-benzylamine as substrates.

The effects of mexiletine were measured *in vitro* by addition to aliquots of the tissue homogenates either 20 min before or at the same time as the addition of substrate. All incubations were carried out in an atmosphere of oxygen at 37°C, and repeated at least 5 times.

When 5-HT, a substrate for MAO-A alone in rat heart, liver and brain was used, double reciprocal plots of initial velocity against substrate concentration with 0, 5, 15 and 50 µM of mexiletine intersected at the same point on the velocity axis. Dixon analysis confirmed that the inhibition produced was competitive with K_i values of 5.5, 5.0 and 3.6 µM for heart, liver and brain respectively. Mexiletine also competitively inhibited the deamination of tyramine by all three tissues.

When benzylamine was used as substrate, mexiletine competitively inhibited its deamination by the heart but not by the liver, where benzylamine is metabolized by MAO-B alone. The same pattern of inhibition was also seen with β-phenylethylamine. Here, mexiletine competitively inhibited the deamination of β-phenylethylamine by MAO-A in the rat heart, but had no effect on the deamination in either liver or brain where this substrate is metabolized by MAO-B. In all cases, control experiments with d- and l-amphetamine produced closely similar results. Preincubation of the homogenates with mexiletine before the addition of substrate did not increase the magnitude of the resulting inhibition. Preincubation of heart and liver homogenates, before the addition of 5-HT, with mexiletine (10^{-4} M) and pargyline (10^{-5} M) protected the MAO-A in both tissues from irreversible inhibition by over 80%.

Mexiletine appears to be a reversible competitive inhibitor that is selective for MAO-A in rat heart, liver and brain, as defined by its substrate-specificity. In this respect mexiletine closely resembles amphetamine.

Supported by a grant from the British Heart Foundation. My thanks are due to Boehringer Ingelheim, Ltd., for generous supplies of mexiletine.

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A model to test the relative potencies of phosphodiesterase inhibitors in brain (*in vivo*)

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Phosphodiesterase occurs in high concentrations in brain (Klainer, Chi, Friedberg, Rall & Sutherland, 1962) and provides the major route for the breakdown of cAMP (Butcher & Sutherland, 1962). Adenylate cyclase has been postulated to be a part of the amine receptor (Rodbell, 1971) and in consequence one would expect enhancement of aminergic mechanisms in the CNS by the use of drugs which inhibit phosphodiesterase. Many drugs possess such properties and have been found to be potent *in vitro* inhibitors. The brain, however, presents specific physical barriers to drugs depending on their structure and hence it is important to assess their potency *in vivo*.

To this end groups of 3 male albino Wistar rats were lightly anaesthetized and 20 µl of [^{14}C]-cAMP (specific activity 278 mCi/m mol) injected intraventricularly by the method of Noble, Wurtman & Axelrod (1967) and killed at 0, 1.5, 3, 6, 15, 30, 60 and 120 min after the injection. The brain was removed rapidly and placed in liquid nitrogen. The [^{14}C]-cAMP content was determined by a modification of the combined methods of Schultz & Daly (1973) and Krishna, Weiss & Brodie (1968). A decay curve for the injected [^{14}C]-cAMP was obtained. Animals in groups of 6 were then pretreated for 30 min with 2.5, 5, 10, 20 and 40 mg/kg i.p. of the phosphodiesterase inhibitor, ICI 63197 (Nahorski & Rogers, 1975) and killed in pairs at 8, 12 and 20 min as indicated to be optimum by the decay curve. To compare all these results a variance analysis was used splitting the data in smaller groups when the interaction between the involved factors was significant. To study the potency of the phosphodiesterase inhibitory effect compared with control and ICI 63197 the means were compared using the Student *t* test when the variance analysis was significant ($P < 0.05$). All doses of ICI 63197 gave a significant increase in [^{14}C]-cAMP ($P < 0.01$ between doses—analysis of variance, and $P < 0.05$ at lowest dose compared with control, Student *t* test). Other drugs (diazepam, theophylline, trifluoperazine and desipramine) were compared on a similar schedule with ICI 63197. Both diazepam (2.5, 5 and 10 mg/kg i.p.) and theophylline (5, 10 and 30 mg/kg i.p.) showed evidence of phosphodiesterase inhibitory activity. Diazepam significantly increased the concentration of [^{14}C]-cAMP with increasing dosage ($P < 0.01$ analysis of variance) but was only significantly different from control at 10 mg/kg ($P<0.01$ Student *t*). It was significantly less potent than ICI 63197 ($P<0.01$ analysis of variance) and a 10 mg/kg dose of diazepam was equipotent with a 5 mg/kg dose of ICI 63197. The theophylline effect also increased with dose ($P<0.01$ analysis of variance). It was less potent than ICI 63197 ($P<0.01$ analysis of variance), both 20 mg/kg and 40 mg/kg giving the same difference from control values as 10 mg/kg of ICI 63197.

Desipramine (3.75, 7.5 and 15 mg/kg i.p.) and trifluoperazine (1.25, 2.5 and 5 mg/kg, i.p.) both of which have been shown to have phosphodiesterase inhibiting properties *in vitro* (Janiec, Korczak-Dziuba & Herman, 1974) showed the same potency as the lowest dose used of ICI 63197 but showed no dose response relationship.

The model appears to be a convenient one to evaluate the *in vivo* potency of potential central phosphodiesterase inhibition.

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The effect of chronic administration of D-penicillamine on the rat pancreas

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In response to secretin the exocrine pancreas secretes a watery juice rich in bicarbonate. There is evidence to suggest that this secretion is produced by ductular elements (Bencosme & Lechago, 1971) which comprise less than five per cent of the mass of the gland (Bolender, 1974).

Feeding rats a copper deficient diet containing D-penicillamine (2 g/kg) selectively destroys pancreatic acinar tissue whilst leaving ductular elements functionally intact (Folsch & Creutzfeldt, 1976). After six weeks on this diet weaning rats exhibited a reduced rate of growth and a much reduced pancreas. Light microscopy indicated a non-inflammatory atrophy and fatty infiltration of pancreatic acinar tissue. Ductular elements were unaffected.

Amylase activity was markedly decreased in pancreatic homogenates from penicillamine treated animals whereas the activities of Mg^{2+} and Ca^{2+} stimulated adenosine triphosphatases (ATPases) together with succinic dehydrogenase (SDH) increased (Table 1). The ratio of the mean enzyme activities in penicillamine treated and control animals was about the same for Mg^{2+}-ATPase (2.7), Ca^{2+}-ATPase (3.2) and the mitochondrial marker SDH (2.6), suggesting that the elevated ATPase activities could have resulted from a relative increase in the concentration of mitochondria in the tissue.

The hormone responsiveness of the duct cells was investigated by determining the effects of secretin on cyclic AMP levels *in vitro*. In unstimulated glands the cyclic AMP levels for control and penicillamine treated animals were 4.7 ± 0.6 (24) and 23.8 ± 6.0 (24) µmoles cyclic AMP/kg protein ($P<0.004$) and in the presence of secretin (0.25 C.U./ml) 10.9 ± 1.2 (24) and 77.8 ± 10.1 (24) µmoles cyclic AMP/kg protein ($P<0.0005$). The magnitude of this stimulation was 2.3 and 3.3 times in control and penicillamine treated animals respectively, indicating that the sensitivity of the gland to secretin is not decreased by penicillamine treatment.

Table 1 Enzyme activities in pancreatic homogenates from control and penicillamine treated rats The values indicated are the mean ± s.e. mean (no. of animals). For all enzymes the control and treated activities are significantly different, $P \leqslant 0.005$ (Mann-Whitney U test)

Enzyme	*Control*	*Penicillamine treated*
Amylase (i.u./mg DNA)	35,268.6 ± 5361.7 (7)	118.9 ± 43.6 (23)
Succinic Dehydrogenase (μmoles Formazan min^{-1} mg DNA^{-1})	162.4 ± 32.5 (7)	415.3 ± 60.4 (23)
ATPases (μmoles Pi min^{-1} mg DNA^{-1})		
(1) Mg^{2+}-ATPase	19.3 ± 2.2 (7)	51.5 ± 7.7 (23)
(2) Ca^{2+}-ATPase	19.5 ± 2.4 (7)	62.2 ± 8.5 (23)

It is concluded that this preparation may prove a useful model for the study of pancreatic duct cells.

Financed by the Cystic Fibrosis Research Trust.

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Inhibition of rabbit muscle pyruvate kinase by lithium

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Lithium is of importance in the prophylactic treatment of manic-depressive psychoses but its exact mode of action is not known. It has been suggested that an important aspect of its action is via interaction with magnesium dependent enzymes (Birch, 1974). Lithium was originally shown to inhibit pyruvate kinase by Kachmar & Boyer (1953) though the level of lithium used was very high (100 mM). A preliminary report of lithium's action on a number of magnesium dependent enzymes has been made (Birch, Hullin, Inie & Leaf, 1974).

The present work was undertaken to provide a detailed investigation of the inhibition of pyruvate kinase by lithium. The interaction of lithium with all the substrates of the enzyme was examined and the results indicate that lithium was competitive with respect to ADP binding to the enzyme and non-competitive with respect to all other substrates. Under the normal assay conditions (85 mM Tris.HCl buffer, pH 7.5, 0.5 mM phosphoenolpyruvate, 10 mM magnesium chloride, 5 mM ADP, 20 mM potassium chloride, 0.25 mM NADH and excess lactate dehydrogenase) 10 mM lithium produced a 16–24% inhibition of the maximum pyruvate kinase activity. This is a lower level of inhibition than indicated by the previous preliminary study (Birch *et al.*, 1974) but is still at a significant level. At the intracellular ADP concentrations of approximately 1.2 mM the level of inhibition is markedly increased. Further work to discriminate between a specific lithium, general ion, or ionic strength phenomena showed that it was not a general ion nor ionic strength effect but that inhibition was also found with calcium and sodium, the order of inhibition being calcium > lithium > sodium. Kinetic investigation showed that the calcium inhibition was of a similar type to lithium, i.e. competitive to ADP binding. However calcium levels unlike lithium levels are rigidly controlled by the body and so the inhibition of pyruvate kinase by calcium is unlikely to be of physiological significance.

The prophylactic dose of lithium gives a plasma lithium level of 0.6–1.4 mM (Hullin, McDonald & Allsopp, 1972) and at this level the inhibition of pyruvate kinase is insignificant. There is still the possibility however of accumulation of lithium in certain body tissues. Should the level be raised to 4 or 5 mM the level of inhibition would be significant especially at the intracellular ADP level. Whether these conditions of 4–5 mM lithium are likely is open to question.

There might be other magnesium dependent enzymes which are more sensitive to lithium levels than pyruvate kinase and we are looking at this possibility.

This work was supported by the Medical Research Council.

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Tissue variability and some properties of the accumulation of [^{3}H]-corticosterone by isolated organs

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Administration of corticosteroid hormones enhances the responsiveness of a number of tissues to agonist drugs and to nerve stimulation (Brodie, Davies, Hynie, Krishna & Weiss, 1966; Gibson, Pollock & Spence, 1976). This supersensitivity may be due to a redistribution of Na$^+$ ions within the tissue, probably as a result of hormone-induced enzyme synthesis (Gibson & Pollock, 1976; Scott & Sapirstein, 1975). The first step in the action of corticosteroids on a target organ is entry of the hormone into the cell (Gorski & Gannon, 1976). However, the entry process is poorly defined, and differences in steroid uptake by various tissues have been reported (Jensen & Jacobson, 1960). We therefore began an investigation of the action of corticosteroids on cellular reponsiveness by observing the accumulation of [^{3}H]-corticosterone in various isolated tissues.

Animals were killed by stunning and exsanguination. The tissues were rapidly dissected and incubated at 37°C. in Krebs bicarbonate solution containing [^{3}H]-corticosterone (70 nM; 112 Ci/mmole; Amersham). The medium was gassed with 95% O_2 5% CO_2. At various time intervals tissues were removed, blotted, weighed and digested in 1 ml potassium hydroxide (0.5 M; 60°C). The radioactivity in a 0.1 ml aliquot of digestant was then measured using a toluene-Triton scintillation fluid.

Initially accumulation of [^{3}H]-corticosterone was observed in the rat anococcygeus muscle. In this tissue, equilibrium was reached within 30 min, and the tissue/medium ratio after 2 h was 2.2 ± 0.07 ($n=6$). However, there was a marked tissue variability in accumulation, the tissue/medium ratios for other tissues following a 2 h incubation being: rat heart (2.5 ± 0.2); mouse heart (3.7 ± 0.05); rabbit vas deferens (3.4 ± 0.3); mouse vas deferens (7.4 ± 0.3); rat pituitary (5.6 ± 0.3); rat hypothalamus (2.6 ± 0.09). The highest tissue/medium ratio was achieved in the mouse vas deferens and this tissue was used to study some further characteristics of the accumulation process.

Dichloromethane extraction of incubated tissues suggested that 96% of extractable radioactivity was unchanged corticosterone. The accumulated steroid was well retained by the vas, the time of washout being 3 times that of accumulation. The accumulation was temperature sensitive being reduced by 32% at 20°C and by 51% at 4°C. Reduction of the Na$^+$ content of the medium to 25 mM enhanced accumulation. Rather surprisingly, accumulation was also enhanced by ouabain (10^{-4} M). Preliminary experiments suggest that part of the accumulation is mediated by a specific process since it could be reduced by excess corticosterone (10^{-4} M) but not by hydrocortisone (10^{-4} M).

In conclusion, the accumulation of [^{3}H]-corticosterone exhibited a marked tissue and species variation. Indeed, in the case of cardiac and smooth muscle this variation exhibited a marked similarity to that described for extraneuronal accumulation of catecholamines (Gillespie & Muir, 1970). The mechanisms responsible for the accumulation and its relation to cellular responsiveness is under investigation.

CVS is an M.R.C. Student

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Two separate cholinergic mechanisms for regulation of oxytocin release

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The oxytocinergic neurones of the lactating rat generate every 5–15 min during suckling a synchronized and brief (2–4 s) burst of action potentials (Lincoln & Wakerley, 1975). Each period of accelerated firing (> 40 spikes/s) discharges a pulse of oxytocin (0.5–1.0 mU) from the neurohypophysis; this circulates to the mammary gland and initiates the ejection of milk. The hypothalamic neurones of the supraoptic and paraventricular nuclei, which synthesize and release oxytocin, are excited by iontophoretically applied acetylcholine (Dreifuss & Kelly, 1972) and cholinomimetics stimulate the release of neurohypophysial hormones (Kuhn & McCann, 1971). It is possible, therefore, that acetylcholine may be an essential synaptic transmitter in the pathway for reflex milk ejection. This was examined by the application of cholinolytics to rats that were reflexly milk ejecting, under urethane anaesthesia.

Lactating rats (250–350 g), separated from their young for 20 h at 8–10 days post-partum, were anaesthetized with urethane (1.2 g/kg, i.p.). Intramammary and arterial pressures were recorded, as described elsewhere (Tribollet, Clarke, Dreifuss & Lincoln, 1977). Three hours later, whilst the animal was still anaesthetized, a hungry litter of young were applied to the nipples. The first milk ejection occurred after 20–40 min, and thereafter milk ejections recurred approximately every 6 minutes. Cholinolytics (atropine, hyoscine, mecamylamine and hexamethonium) were injected into the saphenous vein, in a volume of about 0.3 ml. Cholinomimetics (carbachol and bethanecol) were injected into the lateral cerebral ventricle in a volume of 1 μl; the solutions were made isotonic with NaCl.

Atropine and hyoscine, muscarinic antagonists, failed to abolish the milk-ejection reflex of the rat when given in doses up to 100 mg/kg (0/22). Mecamylamine (0.5–2.0 mg/kg) and hexamethonium (5–10 mg/kg), nicotinic antagonists, caused a substantial delay in the recurrence of milk ejection (27/45). This inhibition was dose-dependent and recovery was obtained.

Both the cholinomimetics, carbachol (0.01–0.2 μg) and bethanecol (0.2–4.0 μg), when injected into the lateral ventricle caused a large prolonged release of both oxytocin and vasopressin, as observed through changes in intramammary and blood pressures (36/46). This release was abolished by atropine (0.1–1.0 mg/kg) applied systemically (11/11). Mecamylamine had no effect at 5 mg/kg (0/8).

A direct action of these drugs on the neurohypophysis or mammary glands was unlikely for the responses to intravenous injections of oxytocin and to endogenous oxytocin released by electrical stimulation of the neurohypophysis remained unaltered. Thus, the milk-ejection reflex of the rat appears to contain an excitatory cholinergic relay of the nicotinic type, placed somewhere other than at the level of the oxytocinergic neurone. By contrast, the response to intraventricular cholinomimetics is muscarinic in type.

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Effect of calcium on acid secretion by the isolated rat gastric mucosa

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Changes in plasma Ca^{++} levels have been shown to affect gastric acid secretion in man and other species (Barreras, 1973). These effects may be caused indirectly via hormonal mechanisms or by a direct action on the parietal cells. We have therefore investigated the effects of various external Ca^{++} concentrations on secretory responses to histamine and to pentagastrin using the isolated rat gastric mucosa (Hearn & Main, 1975).

Ca^{++} concentrations were adjusted over the range of 0.0 to 7.2 mM, in both mucosal and serosal solutions, keeping other cations constant. Drugs were added serosally and acid output recorded via an electrode in the unbuffered mucosal solution. An initial control response (in 3.6 mM Ca^{++}) was compared with the mean of two responses, 90 min apart, in the presence of altered Ca^{++}.

When the control conditions (3.6 mM Ca^{++}) were maintained throughout the 450 min experimental period, a progressive decrease in basal secretion rate was seen. This trend was increased by 7.2 mM Ca^{++} and reversed by 0.9 or 0.0 mM Ca^{++}. The effects were readily reversed on washing with 3.6 mM solutions.

Ca^{++} produced similar effects on acid responses to histamine (25×10^{-6} M, 45 min contact). In control experiments, the responses increased slightly ($+0.55 \pm 0.46$ µmol $cm^{-2}\,h^{-1}$, mean change ± s.e. mean), while lowering Ca^{++} to 1.8 or 0.9 mM produced larger changes ($+3.68 \pm 0.41$, $P < 0.002$ and $+4.67 \pm 1.04$, $P < 0.01$ respectively). In the presence of Ca^{++}-free solutions, with EDTA (0.5 mM) in serosal solution only, test responses were not significantly altered from control ($+0.42 \pm 0.84$, $n = 8$ for all groups).

Using the same experimental design, consistent responses to pentagastrin (1.8×10^{-8} M, 30 min contact) were obtained. Control responses to pentagastrin and histamine did not differ significantly (1.18 ± 0.24, $n = 25$ and 2.01 ± 0.47, $n = 32$, respectively). Under control conditions, there was a mean fall in the test responses to pentagastrin of -0.17 ± 0.08 µmol $cm^{-2}\,h^{-1}$ with a larger decrease of -0.61 ± 0.35 being produced by 7.2 mM Ca^{++} ($n = 6$). A significant increase in size of responses was observed with both 0.9 mM and Ca^{++}-free solutions ($+0.95 \pm 0.29$, $P < 0.05$, $n = 6$ and $+1.04 \pm 0.15$, $P < 0.002$, $n = 7$ respectively).

Raising Mg^{++} concentrations from 1.2 to 2.4 and 4.8 mM (keeping Ca^{++} constant at 3.6 mM) caused a potentiation of responses to histamine, the results being significant only for 2.4 mM solutions ($+4.97 \pm 1.79$, $P < 0.05$, $n = 5$).

These results demonstrate that, in the rat *in vitro*, lowering external Ca^{++} increases both basal and stimulated acid secretion by a direct effect on the secretory mucosa. They provide little support for the hypothesis that pentagastrin acts via Ca^{++}-dependent histamine release within the mucosa, since changing the Ca^{++} concentration had similar effects on the responses to histamine and pentagastrin.

J.B.P. is an M.R.C. Student.

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The effects of some gastrointestinal hormones and metoclopramide on cardiovascular dopamine receptors

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During investigations of the pharmacology of metoclopramide, a drug known to accelerate gastric emptying and stimulate intestinal motility in animals (Jacoby & Brodie, 1967) and man (James & Hume, 1968), we have considered the possibility of an interaction between metoclopramide and various hormones which affect gastrointestinal motility. Metoclopramide blocks cardiovascular dopamine receptors (Day & Blower, 1975) and some actions of secretin may be mimicked by dopamine (Furuta, Hashimoto, Iwatsuki & Takeuchi, 1973). We have therefore investigated the effects of secretin, glucagon, cholecystokinin and the synthetic peptide pentagastrin on cardiovascular dopamine receptors in anaesthetized rats.

Anaesthetized male Wistar rats were prepared as previously described (Day & Blower, 1975). After α- and β-adrenoceptor blockade, the intravenous

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administration of dopamine (0.1 mg/kg), secretin (0.2 u/kg), glucagon (0.5 μg/kg) or cholecystokinin (0.15 u/kg) elicited short-lived falls in blood pressure. These depressor responses were similar in magnitude and duration to those of dopamine. Responses to secretin and glucagon were rapidly tachyphylactic, but this effect was reversed following administration of dopamine and could be prevented by alternating doses of dopamine with doses of either hormone. Responses to cholecystokinin did not show tachyphylaxis but did reduce the responses to subsequent doses of dopamine, secretin or glucagon.

Pentagastrin (0.1 μg/kg) evoked short-lived increases in blood pressure. The hypotensive responses to dopamine, secretin, glucagon and cholecystokinin were completely reversed following administration of pentagastrin (0.1 μg/kg) or metoclopramide (5 mg/kg).

These results suggest that secretin and glucagon may have direct or indirect agonist actions at dopamine receptors. Cholecystokinin appeared to behave as a partial agonist, whilst both pentagastrin and metoclopramide acted as dopamine receptor antagonists. The interaction of these hormones with dopamine receptors could be of importance in elucidating some of their effects on vascular haemodynamics or on gastrointestinal motility.

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The effects of histamine and selective histamine receptor agonists on the isolated working guinea-pig heart preparation

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We have recently described an isolated guinea-pig heart preparation capable of sustaining external work (Flynn, Gristwood & Owen, 1977). Using this preparation the effects of histamine and the selective histamine H_1- and H_2-receptor agonists, 2-pyridylethylamine (Durant, Ganellin & Parsons, 1975) and dimaprit (Parsons, Owen, Ganellin & Durant, 1977) respectively, on cardiac function were investigated.

Full dose response curves for each agonist were constructed in separate preparations. Parallel line assays were used to compare the potencies of 2-pyridylethylamine and dimaprit with histamine. The analysis of variance used to estimate potency showed that the dose-response curves to each of the agonists were parallel over the dose-range used.

Control values for each measured parameter after stabilization of the preparations were: external work (E.W.) 0.410 ± 0.017 kg-m min^{-1} g^{-1}, maximum left ventricular pressure (L.V.P. max.) 84.5 ± 1.2 mmHg, contractility as indicated by $\text{dL.V.P.}/dt$ max. (dp/dt max.) 2239 ± 96 mmHg/s, sinus rate (S.R.) 216.2 ± 4.4 beats/min, aortic flow (A.F.) 360.0 ± 13.8 ml min^{-1} g^{-1}, coronary flow (C.F.) 73.0 ± 4.1 ml min^{-1} g^{-1} and cardiac output (C.O.) 433.1 ± 16.8 ml min^{-1} g^{-1}, ($n = 20$). Where appropriate, measurements are expressed per gram dry weight of heart. These and subsequent figures are means ± s.e. mean.

Histamine increased all measured parameters of cardiac function over the dose-range 10^{-9} to 10^{-6} mol. The maximum absolute increase in each parameter, was calculated from the equation $V = (Vm/1 + K)/A$, where V = response, Vm = maximum response, K = dissociation constant of agonist and A = dose of agonist. The increases were: E.W. 0.353 ± 0.026 kg-m min^{-1} g^{-1}, L.V.P. max. 41.5 ± 2.0 mmHg, dp/dt max. 4132 ± 229 mmHg/s, S.R. 122.9 ± 4.9 beats/min, A.F. 152.7 ± 14.6 ml min^{-1} g^{-1}, C.F. 42.9 ± 4.1 ml min^{-1} g^{-1} and C.O. 188.8 ± 11.9 ml min^{-1} g^{-1}, ($n = 8$).

Dimaprit produced similar changes to histamine in all parameters over the same dose-range. The maximum increase in each parameter was: E.W. 0.422 ± 0.035 kg-m min^{-1} g^{-1}, L.V.P. max. 48.4 ± 6.1 mmHg, dp/dt max. 4398 ± 340 mmHg/s, S.R. 122.8 ± 7.8 beats/min, A.F. 174.5 ± 19.6 ml min^{-1} g^{-1}, C.F. 41.6 ± 1.1 ml min^{-1} g^{-1} and C.O. 211.5 ± 20.1 ml min^{-1} g^{-1}, ($n = 6$).

The potencies, with fiducial limits, of dimaprit relative to histamine (100%) for each parameter, were: E.W. 74.4 (57.2–96.8)%, L.V.P. max. 47.0

(29.3–75.5)%, S.R. 70.9 (50.6–99.4)%, A.F. 64.3 (43.1–95.9)%, *dp*/*dt* max. 57.9 (51.3–65.4)%, C.F. 37.2 (22.1–62.4)%, and C.O. 76.9 (51.3–115.3)%.

2-Pyridylethylamine between 10^{-8} and 10^{-6} mol had little effect on sinus rate, increased coronary flow and produced decreases in all other parameters. Doses in excess of 10^{-6} mol and up to 10^{-4} mol elicited increases in all parameters. The maximum increase in each parameter was consistently less than that produced by histamine. For the increases, the potency of 2-pyridylethylamine relative to histamine (100%) on all parameters, was about 0.2%. This could indicate histamine H_2-receptor stimulation, as 2-pyridylethylamine has been shown to have about 0.2% the activity of histamine on a histamine H_2-receptor system (Durant *et al.*, 1975).

The results indicate the importance of histamine H_2-receptors in the mediation of the changes in cardiac function produced by histamine in this preparation. The involvement of histamine H_1-receptors is less clear from this study although the data suggests that they may mediate a selective cardiac depression.

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The effects of four general anaesthetic agents on the regional distribution of cardiac output in the rat

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The use of radioactive microspheres for the measurement of the distribution of cardiac output (CO) is now an established technique (Rudolph & Heymann, 1967; Mendell & Hollenberg, 1971; McDevitt & Nies, 1976). We have used this technique to compare the cardiovascular effects of four general anaesthetics, sodium pentobarbitone (Sagatal, May & Baker), ethyl carbamate (BDH), alphaxalone/alphadolone acetate (Saffan, Glaxo) and ketamine (Ketalar, Parke-Davis) given intraperitoneally.

Carbonized microspheres (15 μ; 3M Co., St Paul, Minnesota) labelled with ^{85}Sr were injected into the left ventricle of male rats (250–400 g) via a cannula passed down the right carotid artery. Cardiac output and its distribution were determined by the technique of McDevitt & Nies (1976). The results for each anaesthetic are shown in Table 1.

There was no significant difference in the mean cardiac output and arterial blood pressure with the four anaesthetics. Ethyl carbamate produces the most strikingly different distribution of cardiac output when compared to the other three anaesthetics. It produces significant ($P < 0.05$) reductions in flow to the kidneys, spleen, gastro-intestinal tract and in the total hepatosplanchnic flow. Ethyl carbamate also showed a significant increase ($P < 0.05$) in flow to muscle when compared with pentobarbitone and alphaxalone/alphadolone.

Ketamine when compared to the other three anaesthetics produced an increase in flow to the brain. In seven of the animals anaesthetized with ketamine, blood flow to the cerebellum and brain stem, and to each cerebral hemisphere was determined separately. Blood flow to the cerebellum and brain stem was 1.17±0.05 (7), left hemisphere 1.74±0.17 (7) and right hemisphere 1.55±0.13 (7) ml min^{-1} g^{-1}. Thus ligation of the right carotid artery, an integral part of the technique does not significantly reduce flow to the right hemisphere.

Our results indicate that only ethyl carbamate produces a greatly different pattern of distribution of cardiac output compared to the other anaesthetics studied. This may be a consequence of hypersecretion of adrenaline produced by this anaesthetic (Spriggs & Stockham, 1964).

This work was supported by grants from I.C.I. Ltd and Mersey Regional Health Authority. We wish to thank Mr P.J. Roberts for invaluable technical assistance.

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Table 1 Comparison of the effects of general anaesthetics on blood pressure, cardiac output and its distribution

	Sodium pentobarbitone (50 mg/kg)	*Ethyl carbamate (1.4 g/kg)*	*Alphaxalone/ alphadolone (96 mg/kg)*	*Ketamine (120 mg/kg)*
Mean arterial pressure (mmHg)	117.2 ± 4.22 (10)	113.0 ± 8.63 (10)	116.3 ± 3.63(8)	118.6 ± 3.6 (12)
Cardiac output (ml/min)	72.0 ± 7.1 (11)	70.5 ± 7.4 (10)	70.3 ± 8.4 (9)	72.0 ± 5.4 (12)
Cardiac output (ml min^{-1} kg^{-1})	223.2 ±24.5 (11)	240.0 ±25.9 (12)	253.3 ±35.4 (9)	202.5 ±16.9 (12)
% Cardiac output				
Heart	10.0 ± 1.9 (11)	9.6 ± 1.3 (12)	9.1 ± 1.5 (8)	7.0 ± 0.9 (12)
Lungs	3.9 ± 1.1 (11)	7.7 ± 1.7 (12)	2.9 ± 0.5 (9)	4.1 ± 0.7 (12)
Liver*	4.1 ± 0.7 (11)	2.8 ± 0.4 (12)	2.6 ± 0.3 (9)	2.7 ± 0.5 (12)
Spleen	1.3 ± 0.2 (11)	0.4 ± 0.1 (12)	1.3 ± 0.2 (9)	1.1 ± 0.3 (12)
Kidneys	15.4 ± 1.5 (11)	8.0 ± 1.2 (12)	14.8 ± 1.6 (9)	19.0 ± 1.5 (12)
Epididymides	0.23 ± 0.09 (5)	0.15 ± 0.02 (6)	0.21 ± 0.02(6)	0.20 ± 0.01 (11)
Testes	1.1 ± 0.2 (5)	1.3 ± 0.2 (6)	1.3 ± 0.1 (6)	1.0 ± 0.1 (11)
Brain	2.1 ± 0.2 (7)	2.4 ± 0.4 (7)	2.3 ± 0.2 (9)	3.9 ± 0.4 (12)
Gastrointestinal tract	19.2 ± 1.5 (11)	11.8 ± 1.2 (12)	17.4 ± 1.7 (8)	17.1 ± 1.5 (12)
Hepatosplanchnic**	24.5 ± 1.4 (11)	15.0 ± 1.3 (12)	21.1 ± 1.8 (8)	20.1 ± 1.9 (12)
ml min^{-1} g^{-1}				
Skin	0.07 ± 0.012(11)	0.053 ± 0.014(11)	0.11 ± 0.22(9)	0.078 ± 0.009(12)
Skeletal muscle (fore limb)	0.12 ± 0.031(11)	0.33 ± 0.047(12)	0.21 ± 0.03(9)	0.23 ± 0.03 (12)

* Hepatic artery

** Hepatic artery, spleen and gastro-intestinal tract.

All values are given as mean ± s.e. mean with the number of determinations in parentheses.

The relationship between pre-synaptic α-adrenoceptors, stimulation frequency and calcium

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The pre-synaptic α-adrenoceptor feedback mechanism controlling noradrenaline (NA) release shows frequency dependence and is maximally effective at low rates of stimulation (Starke, Endo & Taube, 1975; Dubocovich & Langer, 1976). Various drugs, which we have previously suggested to act via this mechanism, have now been studied to see if they exhibit frequency dependence.

Responses of the mouse vas deferens were elicited by field stimulation using pulses of 2.0 ms, 64 V and frequencies of 0.2, 1.0, 5.0, 10 and 16 Hz. Agonists which stimulate pre-synaptic α-adrenoceptors such as clonidine and noradrenaline (Marshall, Nasmyth, Nicholl & Shepperson, 1977) or tyramine which acts by releasing endogenous noradrenaline (Axelrod, Gordon, Hertting, Kopin & Potter, 1962) all inhibit the twitch and this is inversely proportional to the rate of stimulation. Clonidine (5.6 nM), noradrenaline (3 µM), and tyramine (20 µM) inhibited the twitch at 0.2 Hz by 78%, 79% and 68% respectively and this was reduced at 16 Hz to only 2%, 18% and 2% respectively.

Antagonists at the pre-synaptic α-adrenoceptor, yohimbine (128 nM), phenoxybenzamine (15 µM) and phentolamine (10 µM) potentiated the twitch and this was greatest at low rates of stimulation. However with all the antagonists the maximal effect occurred at 1.0 Hz and not at 0.2 Hz as with the agonists.

The pre-synaptic regulation of NA output may be inversely proportional to the influx of calcium ions. Therefore, at high rates of stimulation the influx of calcium is assumed to be so large that pre-synaptic α-adrenoceptor regulation is ineffective (Starke, Taube & Borowski, 1977; Langer, 1977). To test this possibility the effect of halving the calcium concentration in the Krebs (from 2.5 mM to 1.25 mM) on the twitch and on the effects of clonidine and yohimbine at different rates of stimulation have been studied.

The lowering of the calcium decreased the twitch tension developed at all rates of stimulation ($P<0.05$). If the regulatory effect of pre-synaptic α-adrenoceptor agonists is reduced by the accumulation of calcium at high rates of stimulation, then reducing the concentration of this ion should increase the inhibitory effect of clonidine at all rates of stimulation. This it did most markedly at 10 Hz and 16 Hz where clonidine (5.6 nM) now inhibited the twitch by 53% and 34% respectively compared to 25% and 2% in Krebs containing 2.5 mM calcium. There was also a change in the potentiation of the twitch produced by yohimbine. Halving the calcium concentration shifted the yohimbine (128 nM) frequency-effect curve to the right with the maximum increase in twitch height now occurring at 5 Hz instead of 1 Hz.

All the agonists and antagonists showed frequency-dependence supporting earlier evidence suggesting a pre-synaptic mode of action. The hypothesis that high calcium influx reduces the effectiveness of this regulatory mechanism was supported by experiments in which halving of the calcium concentration increased the effectiveness of clonidine and yohimbine at high rates of stimulation.

NBS is an MRC student.

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Ergometrine: a pre-synaptic α-adrenoceptor agonist

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The ergot alkaloid ergometrine which has no post-junctional α-adrenoceptor antagonist activity (Brown & Dale, 1935) was found to inhibit the twitch response of the isolated mouse vas deferens to electrical stimulation (0.2 Hz, 2.0 ms, 64 V). This action was rapid in onset, dose related (10 nM–10 µM) and readily reversed by washing with Krebs solution. The mechanism of this inhibitory effect has been investigated.

The twitch response of the mouse vas deferens is inhibited by the indirectly acting amine tyramine (Marshall, Nasmyth & Shepperson, 1977a). This effect is prevented by cocaine. The ergometrine inhibition is not produced in the same manner as cocaine (10 µM) did not alter the dose-inhibition curve for the ergot alkaloid.

Drugs which inhibit the neuronal uptake of noradrenaline (NA) also reduce the twitch (Marshall, Nasmyth & Shepperson, 1977b). A number of ergot alkaloids may inhibit NA uptake (Pacha & Salzmann, 1970). However, ergometrine (3 µM) which inhibited the twitch by more than 60% did not alter the uptake of (7–^{3}H)–(–)–NA (10 ng/ml; 9.8 Ci/mmol).

Another mechanism by which ergometrine might have produced its inhibition was by stimulation of β-adrenoceptors (Jenkins, Marshall & Nasmyth, 1976). The dose-inhibition curve to isoprenaline (30–300 nM) was abolished by propraolol (200 nM) but it did not alter the ergometrine curve (100 nM–1 µM).

Clonidine inhibits the twitch response of the mouse vas deferens by stimulating pre-synaptic α-adrenoceptors (Marshall, Nasmyth, Nicholl & Shepperson, 1977). Three tests were used to evaluate this possibility for ergometrine. Firstly phenoxybenzamine (15 µM) a dose which blocks pre-synaptic α-adrenoceptors (Marshall *et al.*, 1977b) abolished the inhibition produced by ergometrine. Secondly, the selective pre-synaptic α-adrenoceptor antagonist yohimbine, (Starke, Borowski & Endo, 1975), 10 nM, shifted the ergometrine dose-response curve to the right. Thirdly, the effects of pre-synaptic α-agonists are inversely proportional to the rate of stimulation (Starke, Endo & Taube, 1975). Ergometrine (300 nM) inhibited the twitch by 75% at 0.2 Hz and this was reduced with increasing rates of stimulation to less than 5% at 16 Hz.

These results strongly suggest that ergometrine inhibits the twitch response of the mouse vas deferens by acting like clonidine as an agonist at pre-synaptic α-adrenoceptors supporting the observations of Ambache, Dunk, Verney & Zas (1973).

NBS is an MRC student. We thank Sandoz for supplying the ergometrine.

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Cardiovascular responses to centrally administered adrenaline in spontaneous hypertensive rats

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The involvement of central noradrenergic neurones in the control of blood pressure is well established and both clonidine and α-methyldopa are thought to exert their action via these neurones (Finch, 1974; Finch & Haeusler, 1973). The exact role of specific central adrenergic neurones, which have only recently been demonstrated in the rat brain (Hökfelt, Fuxe, Goldstein & Johansson, 1973) in areas known to be involved in the control of blood pressure, however, remains unclear.

Preliminary studies, in various species, have shown that intracerebroventricular (i.c.v.) injections of adrenaline exert a variable effect on blood pressure (Toda, Matsuda & Shimamoto, 1969; Day & Roach, 1974). Intracerebral injections of adrenaline into various regions of the brain also induce centrally mediated changes in blood pressure (Struyker Boudier & Bekers, 1975). The present study sets out to characterize the cardiovascular activity of icv administered adrenaline in spontaneous hypertensive rats.

In conscious spontaneous hypertensive rats, prepared for direct recording of blood pressure by the method of Popovic & Popovic (1960), adrenaline (1–20 μg ICV) in 10 μl of 0.01 N HCl caused a dose-related fall in blood pressure and heart rate, while i.c.v. injections of vehicle were without effect. Pretreatment with phentolamine (100 μg i.c.v.) did not significantly antagonize the hypotension or bradycardia induced by adrenaline (10 μg i.c.v.), while pretreatment with (±)-propranolol (100 μg i.c.v.) completely abolished the bradycardia and reversed the hypotensive effects of adrenaline (10 μg i.c.v.) resulting in an increase in blood pressure. Moreover, the hypotension and bradycardia induced by adrenaline (10 μg i.c.v.) was only slightly reduced by pretreatment with (+)-propranolol (100 μg i.c.v.), while pretreatment with the active isomer (−)-propranolol (100 μg i.c.v.) abolished the bradycardia and reversed the hypotension. Similar antagonism of adrenaline (10 μg i.c.v.) induced responses was obtained after pretreatment with sotalol (100 μg i.c.v.) and with the selective β_1-antagonist metoprolol (100 μg i.c.v.).

These results indicate that i.c.v. administered adrenaline is capable of consistently inducing hypotension and bradycardia in conscious spontaneous hypertensive rats and that, in this hypertensive model at least, these effects are probably mediated by central β-adrenoceptors rather than central α-adrenoceptors.

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Selective blockade of presynaptic tryptamine receptors by (−)-cocaine and (+)-cocaine

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5-Hydroxytryptamine (5-HT) stimulates the rabbit isolated heart by releasing noradrenaline from the terminal sympathetic nerves (Fozard & Mwaluko, 1976). Transmitter release results from activation of specific receptor sites which in terms of potencies of selective agonists are closely similar to the receptors on the cholinergic nerves of the guinea-pig ileum (Fozard & Mobarok Ali, 1976) originally designated 'M' because of their sensitivity to blockade by morphine (Gaddum & Picarelli, 1957). However, morphine is not a selective antagonist of 5-HT on rabbit heart, and therefore a search was instituted for a more selective antagonist and to determine if the responses to 5-HT on both noradrenergic and cholinergic neurones might be similarly affected.

Rabbit hearts were perfused at constant pressure with Tyrode solution containing atropine (0.5 μg/ml) at 37°C. Right atrial and ventricular tensions and cardiac rate were recorded as previously described (Fozard & Muscholl, 1971). Drugs were given by bolus injection or incorporated into the perfusion fluid. Segments of guinea-pig ileum were set up in Tyrode solution containing methysergide (1 μg/ml).

On the heart, (—)-cocaine antagonized chronotropic responses to bolus injections of 5-HT (0.5–32 μg) over the range 0.5–8 μg/ml. At 0.5 and 2 μg/ml, the curves were shifted to the right in a parallel fashion and there was no depression of the maximum response. At 8 μg/ml the maximum response to 5-HT was markedly depressed. (—)-Cocaine (0.5 μg/ml) had no effect on responses to 1,1-dimethyl-4'-phenylpiperazinium iodide (DMPP; 5–160 μg/ml) although 8 μg/ml caused marked inhibition with depression of the maximum response. (—)-Cocaine (0.5 μg/ml) significantly enhanced responses to noradrenaline (0.01–2.56 μg). pA$_2$ values for the antagonism of 5-HT and DMPP by (—)-cocaine were 6.24 ± 0.08, $n=4$ and 4.95 ± 0.09, $n=3$ respectively. (+)-Cocaine, which does not block noradrenaline uptake, was a more potent antagonist of 5-HT than (—)-cocaine. The pA$_2$ values for the antagonism of 5-HT and DMPP by (+)-cocaine were 6.90 ± 0.07, $n=3$ and 5.02 ± 0.003, $n=3$ respectively. Lignocaine proved to be only a weak antagonist of 5-HT ($\text{pA}_2 = 3.87 \pm 0.09$, $n=3$) and showed no selectivity (pA$_2$ lignocaine against $\text{DMPP} = 4.16 \pm 0.09$, $n=3$). On the ileum treated with methysergide (—)-cocaine antagonized responses to 5-HT over the concentration range 0.5–8 μg/ml. The pA$_2$ value for the antagonism of 5-HT by (—)-cocaine was 6.00 ± 0.14, $n=5$.

Local anaesthetic properties seem unlikely to explain the 5-HT blocking actions of the cocaine isomers since lignocaine lacked potency and specificity as an antagonist of 5-HT. Similarly, the effects cannot be referred to events at the noradrenaline uptake pathway of the terminal fibres since both the (+)- and the (—)-isomers were effective and there was antagonism of 5-HT at cholinergic nerve endings. The data can be interpreted in terms of an interaction of the cocaine isomers with 5-HT at presynaptic tryptamine receptors. Should this be correct then the data provide support for the suggestion that neuronal receptors of the heart and ileum for 5-HT are similar (Fozard & Mobarok Ali, 1976).

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A benzotriazinium salt as a potential antiarrhythmic agent

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Four series of substituted benzotriazinium iodides have recently been synthesized (Stevens & Stevens, 1970). These compounds have many pharmacological properties similar to those of quinidine, notably the effects on cardiac tissue. We have investigated 2-n-propyl-4-p-tolylamino-1,2,3-benzotriazinium iodide (TnPBI) in view of its potential antiarrhythmic properties.

Guinea-pig isolated atrial pairs were mounted on a Sylgard 182 resin base in a channel cut from a perspex block and perfused at 32°C with oxygenated Locke solution of the following composition (mM) NaCl 154, KCl 5.6, CaCl$_2$ 2.16, glucose 5.5, NaHCO$_3$ 2.4. Cells were impaled with glass microelectrodes filled with 3M KCl, and the action potentials were amplified by a Grass P16 DC preamplifier and displayed on an Advance OS 4000 digital storage oscilloscope. Stored action potentials were then drawn out on a pen recorder via an Advance OS 4001 analogue output unit. The atria were stimulated at a frequency of approximately 10% above their spontaneous frequency by means of bipolar platinum electrodes in contact with the surface of the left atrium.

TnPBI increased the duration of the action potential (APD), decreased the maximum rate of depolarization (MRD) and decreased conduction velocity. These effects were dose related between 1×10^{-6} M and 1×10^{-5} M. TnPBI did not significantly alter the resting membrane potential but the size of the overshoot and consequently of the action potential were reduced. With one exception, these effects are similar to those produced by quinidine: the exception is the prolongation of the APD. The work of Vaughan Williams (1958) on rabbit atria is widely quoted, indicating that quinidine does not prolong the APD, although we have found in preliminary experiments that quinidine does, in fact, prolong the APD in guinea-pig atria.

In strips of electrically stimulated guinea-pig ventricles, TnPBI had much less marked effects on the APD, but significantly reduced the MRD and conduction velocity. As in the atria, the resting membrane potential was unchanged, but there was a significant reduction in the magnitude of both the overshoot and the action potential especially at the concentrations of 5×10^{-6} M and 1×10^{-5} M.

At the concentrations used for the intracellular studies, TnPBI reduced the spontaneous rate of beating of guinea pig isolated atrial pairs, but had only slight inhibitory effects on the force of contraction.

It thus appears that TnPBI produces essentially similar effects on guinea-pig cardiac muscle as quinidine, and may deserve further investigation as an antiarrhythmic agent.

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Taipoxin, a presynaptically active neurotoxin, destroys mammalian skeletal muscle

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Taipoxin, isolated from the crude venom of the taipan *Oxyuranus scutellactus* has been defined as a presynaptically active neurotoxin (Kamenskaya & Thesleff, 1974). We have now demonstrated that taipoxin causes a necrotizing myopathy of mammalian skeletal muscle.

Taipoxin (2.0 µg in normal saline in a dose volume of 0.2 ml) was injected into one hind limb of female Wistar rats weighing 160–180 g. The injection was made so that the soleus muscle would be exposed to the toxin but not damaged by the insertion of the needle.

Within 1 h the wet weight of the intoxicated soleus muscles increased by 44% due to the accumulation of fluid within the interstitial spaces. The muscles remained oedematous for about 24 h after the injection of the toxin. During the period 3–10 days after injection, the muscles were wasted; the wet weight returned to normal by 21–28 days. The early increase in muscle wet weight was accompanied by the necrosis and phagocytosis of large numbers of superficial muscle fibres, the deeper fibres remaining undamaged. By 3 days, however small myotubes were present in the muscle. Immature muscle fibres were visible by 7 days and by 21 days the intoxicated muscles were virtually normal except for the persistence of centrally located nuclei.

The degeneration and regeneration of the muscles could be documented using physiological techniques. Thus by 6 h the mean resting membrane potential of the muscle fibres had fallen from a normal value of −76.9 mV (s.e. mean 0.31, $n=90$) to −15.6 mV (s.e. mean 2.1, $n=84$). By 3 days however, the mean resting membrane potential was −58 mV (s.e. mean 1.4, $n=70$) and was 'normal' by 7–14 days. During the period of 3–7 days after intoxication, fibrillation was common and in many fibres action potentials could be generated in the presence of tetrodotoxin (10^{-6} M).

The results demonstrate that taipoxin behaves both as a presynaptically active neurotoxin and as a myotoxin.

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The hepatotoxicity of lithocholic acid in male mice

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A few years ago it was thought that an important therapeutic advance had been made when it was found that oral administration of chenodeoxycholic acid (CDCA) to patients with cholesterol gallstones reduced the cholesterol saturation of bile and caused partial or complete dissolution of the gallstones (Danzinger, Hofmann, Thistle & Schoenfield, 1972). However optimism that this form of therapy would obviate the need for surgical treatment of gallstones in selected patients has declined, and clinical trials of CDCA treatment have been limited to certain centres in many countries (Dowling, Murphy & Iser, 1976). The major reason for caution in this form of therapy is that CDCA (3α,7α-dihydroxy-5β-cholanoic acid) is partially 7α-dehydroxylated by the intestinal microflora to yield the hepatotoxic secondary bile acid, lithocholic acid (3α-hydroxy-5β-cholanoic acid) (LA), some of which returns to the liver during enterohepatic circulation of bile acids. The large and prolonged doses of CDCA (about 1 g per day for many months) required for gallstone dissolution introduce the risk that the amounts of LA formed from the CDCA might cause liver damage in patients. Although LA causes intrahepatic cholestasis and early damage to the bile canaliculi in rodents, the mechanism by which LA produces these effects is not known.

As part of a programme on the effects of sex hormones on hepatobiliary function (Taylor, 1977) we have studied the effects of administering LA to male mice and monitoring the response by determining the cholesterol and bile acids of the gall-bladder bile and observing the development of lesions in the liver by standard histopathological techniques. Mature Balb/c mice were dosed with 8 mg of LA per day by gavage. Control animals received saline only. Ten animals from each group were killed at 0.5, 1.5, 2.5 and 3.5 days after the start of treatment. The gallbladder biles from each group were pooled. Cholesterol was determined colorimetrically, and bile acids (B.A.) by gas chromatography-mass spectrometry (Taylor, 1977). Samples of liver were taken for histological studies. Marked changes were observed only in the

LA-fed animals. Biliary cholesterol concentrations rapidly increased while total B.A. first increased and then decreased, thus causing a dramatic decrease in the total B.A./cholesterol ratio. Between 1.5 and 2.5 days there was a large decrease in cholic acid concentration: LA and its major metabolite $3\alpha,6\beta$-dihydroxy-5β-cholanoic acid, not detectable in bile of control animals, appeared in increasing amounts. These marked changes in bile acids preceded the appearance of liver damage which was well established only after 2.5 days. The livers of LA-treated animals showed areas of necrosis of irregular distribution with some dilatation of centro-lobular venules with inflammatory and neutrophil infiltration. LA may exert its hepatotoxic effect, in mice by alteration in cholesterol and bile acid biosynthesis in the liver, but the sulphating mechanism may reduce potential toxicity in man.

W.T. thanks The Wellcome Trust for a Travel Grant, and the Smith, Kline & French Foundation for a Research Grant.

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Haem biosynthesis and hepatic drug metabolism in lead poisoned rats

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Previous animal studies (Scoppa, Roumengous & Penning, 1973; Alvares, Leigh, Cohn & Kappas, 1972) have produced indirect evidence that the depression in hepatic microsomal cytochrome P-450 content and the mixed function oxidase system in lead intoxicated rats, is associated with depressed haem synthesis. This, however, remains to be proven. The present study therefore investigated the inter-relationship of haem biosynthesis and cytochrome P-450 in lead intoxicated rats.

With increasing pretreatment of rats with intraperitoneal injections of lead, there was a progressive decrease in hepatic microsomal cytochrome P-450 and b_5 content and decreased activity of the enzymes aniline hydroxylase and aminopyrene demethylase. Associated with this impairment of the microsomal mixed function oxidase system there was a depression in haem synthesis, as assessed by decreased activity of the enzymes δ-aminolaevulinic acid (ALA) dehydratase, coproporphyrin oxidase and ferrochelatase whilst the activity of the rate-limiting enzyme of haem biosynthesis ALA synthase was increased. The activity of ALA synthase has been shown to be regulated by free haem levels; thus when free haem levels are depressed the activity of the enzyme is increased by a negative feedback mechanism. A highly significant ($P < 0.001$) inverse relationship ($r = 0.76$) was found between hepatic ALA synthase activity and microsomal cytochrome P-450 content (Figure 1). This indicates that the depression in the levels of the haemoprotein cytochrome P-450 in animals treated with lead is due to impaired haem synthesis resulting in decreased

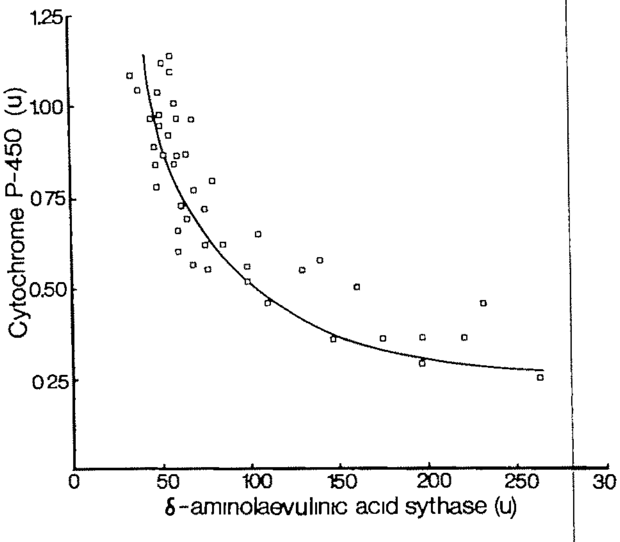

Figure 1 The correlation of hepatic γ-aminolaevulinic acid (ALA) synthase activity with microsomal cytochrome P-450 content ($r = 0.76$; $P < 0.001$), where cytochrome P-450 = (41.4 ± 3.4/ALA synthase) + (0.098 ± 0.034).

availability of haem for synthesis of the haemoprotein and a decrease in the regulatory 'pool' of haem.

The activity of microsomal haem oxygenase was also increased by lead pretreatment. It is therefore concluded that the depression in the microsomal mixed function oxidase system in lead intoxication is the result of depressed haem synthesis and also possibly by an increase in haem degradation.

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The metabolism of bupivacaine in the rat

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Bupivacaine (Marcain, *N*-*n*-butylpipecolylxylidine) is a long-acting local anaesthetic widely used for lumbar epidural anaesthesia in childbirth. It is almost completely metabolized in human volunteers, mothers in childbirth and by their babies (Caldwell, Moffatt, Smith, Lieberman, Cawston & Beard, 1976) but little is known of the pathways of metabolism. Accordingly we have investigated the fate of this drug in the rat as a preliminary to human studies.

[^{14}C]-Bupivacaine hydrochloride, labelled in the carbonyl group, was synthesized, administered to female Wistar albino rats (30 mg/kg; 2.5 μCi/animal) by intraperitoneal injection, and their urine and faeces collected for four days. The excretion of [^{14}C] was monitored by liquid scintillation counting, and 74% of the dose was recovered (33% urine: 41% faeces). The bulk of the [^{14}C] excreted (urine 27%, faeces 29%) appeared on the first day.

Urinary metabolites were examined by thin-layer chromatography followed by radiochromatogram scanning, reverse isotope dilution, gas liquid chromatography, gas chromatography-mass spectrometry (g.c.-m.s.) and u.v. spectrometry. Five urinary metabolites were identified, together with two unknowns, and their identities and quantities are given in Table 1. The major routes of metabolism of bupivacaine in the rat involve hydroxylation of the aromatic ring to yield two isomeric phenolic metabolites, 3′- and 4′-hydroxybupivacaine, excreted free and as glucuronic acid conjugates, the ratio of free to conjugated material being about 0.25 in both cases. Standard compounds were not available, and the g.c.-m.s. properties of these metabolites do not permit unequivocal assignment of the position of the hydroxyl group and the assignment of these metabolites is by analogy with those of local anaesthetics of similar structure (see Dring, 1976). *N*-dealkylation to yield desbutylbupivacaine was a minor pathway, as was hydrolysis of the amide bond giving pipecolic acid. Two unknown minor metabolites were also separated but not characterized, and a small amount of bupivacaine was excreted unchanged.

The fate of bupivacaine in the rat is similar to that of other xylidide local anaesthetics, such as lignocaine and mepivacaine, especially in the predominance of aromatic hydroxylation (see Dring, 1976). However, the extent of *N*-dealkylation and amide hydrolysis is much lower than in the case of lignocaine, probably due to the size of the *N*-*n*-butyl substituent and to steric factors associated with the pipecolic acid moiety.

Table 1 Urinary metabolites of bupivacaine in the rat

Compound	*% Urinary ^{14}C as that compound*
Bupivacaine	3.4
Desbutylbupivacaine	1.1
3′-Hydroxybupivacaine	
free	8.9
glucuronide	36.4
4′-Hydroxybupivacaine	
free	5.6
glucuronide	22.7
Pipecolic acid	6.0
Unknown 1 (neutral)	2.2
Unknown 2 (acidic)	13.1

0–24 h urine of rats dosed with [^{14}C]-bupivacaine was analysed as described. Figures are the means of 3 animals.

This work was supported by a grant from Duncan, Flockhart & Co. Ltd. We thank the Wellcome Trust for a grant for inter-disciplinary research.

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Cardiovascular actions of prostacyclin (PGI$_2$) in chloralose anaesthetized dogs

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Prostacyclin (PGI$_2$ or PGX) is an unstable intermediate of arachidonic acid metabolism, which strongly inhibits aggregation of human platelets (Moncada, Gryglewski, Bunting & Vane, 1976) and relaxes several isolated vascular preparations (Bunting, Gryglewski, Moncada & Vane, 1976; Dusting, Moncada & Vane, 1977). Prostacyclin is the predominant active metabolite of the cyclic endoperoxides in all vascular tissues studied, including bovine coronary arteries (Dusting *et al.*, 1977). We have now measured the cardiovascular effects of prostacyclin in anaesthetized open-chest dogs.

Anaesthesia was induced with thiopentone (20–25 mg/kg) and maintained with chloralose (50 mg/kg, i.v.) supplemented (5 mg/kg i.v.) as required. The dogs were artificially ventilated; arterial $P\text{O}_2$ was maintained above 100 mmHg and $P\text{CO}_2$ in the range 28–40 mmHg. Electromagnetic flow probes (Statham Instruments Inc.) were fitted to the ascending aorta (5 dogs) and to the left circumflex artery (8 others). Cardiovascular parameters were recorded as previously described (Hughes, 1971). Mean coronary flow (over 4 s intervals) and coronary vascular resistance were also computed. Drugs were infused into the right femoral vein, into a cannula in the left atrial appendage, and in some experiments via a fine catheter in the left circumflex artery distal to the flow probe.

Intravenous infusion of prostacyclin for 3 min (50–1,000 ng kg^{-1} min^{-1}) caused dose-dependent decreases in systemic blood pressure, total peripheral resistance, and coronary vascular resistance and moderate increases in stroke volume and cardiac output (maximum increase 72 ± 20%, mean ± s.e. mean). Heart rate changes were variable. The stable metabolite of prostacyclin, 6-oxo-PGF$_{1\alpha}$had no effect (up to 10 µg kg^{-1} min^{-1}).

The cardiovascular effects of prostacyclin were similar after intravenous or left atrial infusions. Whereas prostaglandin E$_1$ (left atrial infusions) was slightly more potent than prostacyclin, it was about one tenth as potent when given intravenously.

Intravenous infusion of prostacyclin (50–500 ng kg^{-1} min^{-1}) increased peak phasic coronary flow only at the highest infusion rate. However, mean coronary flow did not change significantly at any infusion rate although coronary vascular resistance was substantially reduced. Direct injection of prostacyclin (50–1000 ng) into the left circumflex artery increased both phasic and mean coronary flow and decreased coronary vascular resistance (for 1–4 min) without any change in systemic blood pressure or heart rate. Similar effects with PGE$_1$ were more persistent (up to 8 min) whereas PGE$_2$ was less potent.

After indomethacin (5 mg/kg i.v.: 4 dogs) or sodium meclofenamate (2 mg/kg i.v.: 2 dogs) there was a slow increase in systemic blood pressure and coronary vascular resistance, and a reduced phasic and mean coronary flow. Indomethacin and meclofenamate potentiated the coronary dilator effects of the smaller intravenous infusions of prostacyclin but did not alter the hypotensive effects. Thus, the sensitivity of the coronary vascular bed *in vivo* is enhanced when endogenous biosynthesis of prostaglandin-like substances is inhibitied.

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Prostacyclin (PGI_2) inhibits the formation of platelet thrombi induced by adenosine diphosphate (ADP) *in vivo*

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An enzyme located in vascular endothelium converts prostaglandin endoperoxides into an unstable substance (prostacyclin; PGI_2) which inhibits ADP-induced platelet aggregation *in vitro* (Moncada, Gryglewski, Bunting & Vane, 1976). It has been suggested that PGI_2 plays an important role in protecting blood vessel walls from platelet deposition.

The formation of intravascular platelet thrombi in the micro-circulation of the hamster cheek pouch was studied using the method of Begent & Born (1970). The everted cheek pouch was superfused at 5 ml/min with a modified Krebs bicarbonate solution at 36°C and micropipettes (tip diameter$=2.5-5.0\ \mu m$), containing ADP (10^{-2} M) were placed with micromanipulators so that their tips were close to the walls of small blood vessels. ADP was applied to the vessel wall iontophoretically with a current between the micropipette and a reference electrode immersed in the superfusing solution.

Currents of up to 500 nA applied to pipettes filled with 0.9% (w/v) saline did not cause formation of thrombi. With ADP-filled pipettes, in the absence of current, platelet thrombi did not appear in the blood vessels. When a current of 200–300 nA was applied, thrombi appeared on the vessel wall close to the tip and at sites downstream from the tip. During continuous application of ADP, thrombi adhered to the vessel walls for up to 3 min before they embolized and were swept away in the blood stream. The embolization of platelet thrombi was filmed at 24 frames/s and a measure of mean blood flow was obtained by analyzing the film (Begent & Born, 1970).

The mean time of ADP application required to induce the formation and embolization of platelet thrombi in venules (30–40 μm in diameter) in which the mean blood flow was 1.0–1.5 mm/s was 20.8 s (±3.2 s.e. mean; range, 4.6–34.1; 8 experiments). Prostacyclin or its stable metabolite, 6-keto $PGF_{1\alpha}$ (Johnson *et al.*, 1976) were dissolved in 50 nM Tris buffer (pH 7.6) and infused at 0.05 ml/min into the inlet of the superfusing stream (5 ml/min). PGI_2 (1, 10 or 100 ng/ml) caused concentration-dependent increases in the time of ADP application required to induce thrombi formation; with 100 ng/ml, the mean time was 110 ± 24.3 s (6 experiments). 6-Keto $PGF_{1\alpha}$ had no effect at concentrations up to 10 μg/ml. In four experiments when the concentration of PGI_2 in the superfusing fluid was 1 μg/ml, repeated application of ADP for periods of 5 min failed to induce thrombi formation. In these experiments the response to ADP returned to normal 30 min after the infusion of PGI_2 had stopped. Concentrations of PGI_2 (up to 1 μg/ml) or 6-keto $PGF_{1\alpha}$ (10 μg/ml) did not cause a change in the mean diameter of the venules. The highest concentration of PGI_2 at which embolization of thrombi could still be used as a measure of blood flow was 100 ng/ml. At this concentration there were no significant changes in mean blood flow.

Prostacyclin and 6-keto $PGF_{1\alpha}$ were kindly supplied by Dr N. Whittaker of the Wellcome Research Laboratories.

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Modulation of thrombus formation *in vivo* by prostaglandins

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We have previously demonstrated the effects of prostaglandins (PG, E_2, E_1 and metabolites) (Lewis & Westwick, 1976) and PGG_2 (Lewis, Westwick & Williams, 1977) on the diameter of normal arterioles *in vivo*. We have now examined the possibility that arteriolar thrombosis can be locally modulated by prostaglandins produced by blood and vascular tissue. An animal model was developed which allowed quantitation of arteriolar thrombus formation during application of test substances to arterioles.

The hamster cheek pouch (HCP) preparation was set up as described by Lewis & Westwick (1975). Following a 45 min equilibration period, the flow of Krebs solution was switched off for 5 min and an arteriole (40–70 μm diameter) was selected. PG (G_2, D_2, E_2 and E_1) or vehicle was dropped onto the Krebs solution immediately adjacent to the selected arteriole, followed 30 s later by electrical micro-damage (+ve square wave DC pulses via 3 μm glass monopipette (filled 1 M KCl) at 10 Hz, 20 ms and current 25 μA for 2 s) and 1 min later by a similar application of ADP (10^{-6} M final bath concentration). The arteriole was continually observed and thrombus formation was quantitated by measuring the total time during which thrombi were present within a period of 10 minutes. The design of the experiment was such that each vessel received three applications of vehicle, five of test solution and two more of vehicle. Results were expressed as percentage difference from control and are shown in Table 1.

Table 1 Effect of prostaglandin (as a percentage difference from control) on thrombus formation in hamster cheek pouch arterioles

Prostaglandin	*Conc.* (ng/ml)*	*Percentage (mean ± s.e. mean)*	*P*	*Prostaglandin*	*Conc.* (ng/ml)*	*Percentage (mean ± s.e. mean)*	*P*
PGE_1	12.5	−11 ± 8	NS	PGE_2	12 5	+11 ± 35	NS
	125	−39 ± 11	<0.05		125	+39 ± 32	NS
	625	−57 ± 9	<0.05		1250	−14 ± 11	NS
	1250	−97 ± 3	<0.05				
				PGG_2	12.5	−6 ± 27	NS
PGD_2	125	−6 ± 1	NS		125	−13 ± 7	NS
	1250	−16 ± 20	NS		625	−55 ± 14	<0.05

NS = $P > 0$ 05
Conc.* = final bath concentration.

PGE_1 appears to be a potent inhibitor of thrombus formation *in vivo* as first described by Emmons, Hampton, Harrison, Honour & Mitchell (1967). PGD_2, which has been shown to be a potent inhibitor of human platelet aggregation, but ineffective on rat platelets (Smith, Silver, Ingerman & Kocsis, 1974) is also ineffective in preventing thrombus formation in the hamster.

These results indicate that the PGG_2 was converted by the vascular tissue *in vivo* to prostacyclin which produced the inhibition of thrombus formation. Prostacyclin formation might also explain in part the prolonged vasodilator activity observed in a previous study with this preparation (Lewis *et al.*, 1977). Prostacyclin was shown to be generated by vessel wall microsomes *in vitro* and to inhibit platelet aggregation *in vitro* (Moncada, Gryglewski, Bunting & Vane, 1976).

Thus, the effect of PGG_2 on thrombus formation appears to depend on the route of its metabolism. If it is metabolized by the enzyme system of the blood vessels as in the present experiments, it is converted to prostacyclin and inhibits thrombus formation, whereas if it came into contact with platelets it would be converted to thromboxane TxA_2 as shown by Hamberg, Svensson & Samuelsson (1975) and thrombus formation would be potentiated or even initiated.

This work was supported by Ciba-Geigy and the Wellcome Trust. The gift of prostaglandins from Professor B. Samuelsson (Stockholm) and Dr J.E. Pike (Upjohn, Kalamazoo) is gratefully acknowledged.

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Inhibition of bile salt-induced gastric mucosal erosions by 16,16 dimethyl prostaglandin E_2 in the rat

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Exposure of the rat gastric mucosa to bile salt (sodium taurocholate, ST) and concurrent administration of indomethacin may lead to erosion formation. Such erosions can be inhibited by prostaglandins even in the presence of exogenous acid (Whittle, 1976) suggesting that prostaglandins have a protective effect on the mucosa, in addition to their anti-secretory actions.

We have investigated the effects of 16,16 dimethyl PGE_2 (MePG) on erosions induced by ST alone using the rat gastric chamber preparation (Mersereau & Hinchey, 1973). The mucosal solution (10–100 mM HCl made iso-osmotic with mannitol) was replaced every 0.5 h and H^+ loss, Na^+ gain and mucosal blood flow (MBF) (aniline clearance, Main & Whittle, 1973) were measured.

Erosion formation was related to the concentration of ST (present for one 0.5 h period) and acid (present from 2 h before until 2 h after ST). The erosion indices 1.5 h after exposure to 20 mM ST in 10 mM HCl (3 ± 2.5, $n=3$) or 2 mM ST in 100 mM HCl (0, 12, $n=2$) were not significantly different from controls (0 and 5.5 ± 5, for 10 and 100 mM HCl respectively, $n=4$).

With 5 mM ST (100 mM HCl) erosion formation began during the exposure period, rose rapidly in the next period and continued to rise for the remaining 1.5 h of the experiment. H^+ loss (per 0.5 h period) increased from 39 ± 11 to 99 ± 7 µmol ($P < 0.05$, $n=4$) during ST then fell steadily. Na^+ gain increased from 26.6 ± 4 to 39.8 ± 4 µmol ($P < 0.01$) during ST, reached a maximum (65.5 ± 5 µmol, $P < 0.01$) in the next period then declined. MBF was unchanged during ST but rose rapidly in the next period ($237 \pm 9\%$ of basal, $P < 0.001$) and remained significantly elevated.

MePG (15 µg ml) in contact with the mucosa from 0.5 h before until 1 h after ST (5 mM, 100 mM HCl) inhibited erosion formation (index reduced from 44 ± 9 to 6 ± 3, at 1.5 h $P < 0.01$, $n=4$) while a lower concentration (5 µg ml) had no significant effect (32 ± 11, $n=4$).

Although no H^+ loss was noted with the low concentration of MePG prior to ST there was an increased loss (35 ± 4 to 59.5 ± 9 µmol, $P < 0.05$) with the high concentration. However, this latter concentration reduced the increased loss due to ST (from 99 ± 7 to 63 ± 15 µmol, $P < 0.05$). Na^+ gain increased from 17 ± 4 to 43 ± 5 µmol ($P < 0.01$) and 17.2 ± 2 to 36.7 ± 5 µmol ($P < 0.01$) for high and low concentrations respectively during the first period of MePG application but did not increase further during ST. MePG raised MBF to $142 \pm 9\%$ ($P < 0.01$) and $130 \pm 4\%$ ($P < 0.001$) for high and low concentrations respectively but failed to affect the further rise following ST.

These results show that MePG applied topically can protect the mucosa against ST. This protective action was associated with a reduction in the increased mucosal permeability to H^+ caused by ST. Protection is unlikely to result from the marked effects on Na^+ transport or MBF since both concentrations of MePG studied had similar effects on these parameters though the lower concentration did not prevent erosions.

R.M. is an M.R.C. student. The prostaglandin E_2 was kindly provided by Dr J.E. Pike of the Upjohn Company, Kalamazoo.

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The presence of a prostaglandin-like substance in the skin of the plaice, *Pleuronectes platessa* L.

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Fletcher & Baldo (1974) observed that the intradermal injection of fungal extracts which precipitate with human C-reactive protein and normal plaice serum caused an immediate (Type 1) hypersensitivity reaction in the skin of plaice, *Pleuronectes platessa* L. This communication reports the results of experiments designed to identify the pharmacological mediators responsible for this cutaneous anaphylaxis.

Skin from freshly killed plaice was chopped, washed and incubated with a specific plaice ringer (Cobb, Fox & Santer, 1973) in the presence of an extract of the dermatophyte fungus, *Epidermophyton floccosum.* Samples were withdrawn after incubation at room temperature and tested for smooth muscle activity on the following isolated preparations: gerbil and rat colon, rat duodenum and stomach strip and guinea-pig trachea and ileum. The extract contracted all tissues but the rat stomach strip preparation gave the most reproducible and dose-dependent responses and so was used to assay the active principle from the skin in subsequent experiments.

The fungus alone had no smooth muscle stimulating activity but extracts of skin which had not been challenged with the fungus exhibited a low level of activity.

The contractile response of the rat stomach strip to skin extract persisted even in the presence of antagonists of acetylcholine, histamine and 5-hydroxytryptamine and also following incubation with chymotrypsin, which destroys activity due to bradykinin.

Indomethacin (10–1000 μg/kg i.p.) injected into plaice 2 h before killing, produced a dose-dependent inhibition of the release of active material from both challenged and non-challenged skin. The activity could be partitioned from an acid aqueous phase into chloroform, from there into Krebs solution (pH 7) and finally back into chloroform at pH 3. This behaviour is consistent with the properties of a prostaglandin.

Following extraction, characterization of the prostaglandin-like activity was attempted using preparative thin-layer chromatography. In six different solvent systems, the pharmacological activity co-chromatographed almost exclusively with authentic E_2, the remainder (<5%) with prostaglandin F. Release of active material from challenged and non-challenged skin was then assayed by the bracketing technique on the rat stomach strip in equivalents of PGE_2. The extract from challenged skin produced a significantly ($P < 0.05$) greater response with incubation times between 20–80 minutes. There was no significant difference in the responses after 120 min incubation.

An immediate hypersensitivity reaction resembling that induced by *Epidermophyton floccosum* resulted from the intradermal injection of PGE_2 (100 ng/ml). Indomethacin did not however completely inhibit the fungal skin reaction *in vivo,* suggesting that other mediators may also be involved.

We thank Dr J.E. Pike, Upjohn Company, for the prostaglandins used in this study. A.A.A. is supported by the Scottish Education Department.

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Stimulation of platelets and macrophages by carrageenin

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Carrageenin, a sulphated polysaccharide from green algae, induces hypotension when injected intravenously (Di Rosa & Sorrentino, 1970) and subplantar administration evokes a marked inflammatory response (Winter, Risley & Nuss, 1962). Different carrageenins vary in their inflammatory and hypotensive potency (Di Rosa, 1972) but the reasons for this are not clear. Since secretion by macrophages and platelets has been implicated in inflammatory responses, we have compared the activity of 4 carrageenin preparations as stimulants of blood platelets and macrophages *in vitro.*

Macrophage culture medium (CM) consisted of 'Autopow' minimum essential medium (Flow

Laboratories), supplemented with 2 mM glutamine, 20 mM HEPES buffer, 5 mM sodium bicarbonate, 50 µg/ml gentamycin and 20% (v:v) heat inactivated foetal bovine serum. Macrophages were obtained by peritoneal lavage of mice 3–4 days after injection of 1 ml thioglycollate medium. Cells were washed twice with phosphate buffered saline (PBS), resuspended in CM and incubated in multiwell plates at 37°C for 3–4 hours. Dead and non-adherent cells were removed by rinsing twice with PBS and macrophages were maintained in CM. Platelet aggregation and release of 5-hydroxytryptamine (5-HT), lysosomal enzymes (B-N-acetylglucosaminidase) and adenine were measured in 0.1 ml volumes of human platelet-rich plasma (PRP) and plasma-free platelet suspensions as previously described (Corey, Gordon, MacIntyre & Salzman, 1977). Plasma-free platelet suspensions were prepared by gel filtration (Salzman, Lindon & Rodvien, 1976). For estimation of macrophage secretion B-N-acetyl-glucosaminidase activity was measured fluorimetrically as for platelets and lactate dehydrogenase (LDH) activity was measured photometrically (Wroblewski & La Due, 1955).

Gelcarin, Iota, Kappa and Lambda carrageenins (100 µg/ml) stimulated macrophage lysosomal enzyme secretion after 36 h incubation; little release was observed after 14 hours. There was no significant leakage of the cytoplasmic enzyme LDH after 36 hours. The rank order of potency of the carrageenins was Iota > Lambda > Gelcarin > Kappa. All the carrageenins induced platelet aggregation showing the same order of potency as for macrophage secretion. Aggregation in PRP, but not in gel-filtered platelets, was preceded by a large increase in optical density, but since this was also observed in platelet poor plasma it presumably represents an alteration in plasma proteins. The aggregation response in citrated PRP consisted of two phases, the second of which was associated with plasma proteins. The aggregation response in citrated PRP consisted of two phases, the second of which was release of platelet granule constituents. This was a selective process—less than 10% release of the cytoplasmic marker, adenine, accompanied release of 70% 5-HT (dense granule constituent) and 35% B-N-acetylglucosaminidase (α granule constituent).

Aspirin (1 mM) inhibited only the second phase of aggregation and the associated release of 5-HT and B-N-acetyl-glucosaminidase. Both phases of aggregation were inhibited by prostaglandin E_1 (1 µM) and aminophylline (5 mM), which elevate cellular cyclic AMP concentrations; by EDTA (1 mM) and EGTA (1 mM), which chelate divalent cations; and by quinacrine (10–50 µM), which inhibits phospholipase A_2 (Flower & Blackwell, 1976).

Carrageenin—induced platelet aggregation in heparinized PRP and gel-filtered platelets consisted of only one phase which was not associated with secretion of granule constituents.

In summary, the rank order of potency of the 4 carrageenins at stimulating secretion from macrophages and blood platelets *in vitro* was the same, and this is in accord with their relative potency *in vivo* (McCandless, 1965). There appears to be a common structural requirement for carrageenin activity as a secretory stimulant *in vitro* and as an inflammatory agent *in vivo*. Further studies *in vitro* should help to elucidate how inflammatory lesions are induced by carrageenin *in vivo*.

This study was supported by grants from the M.R.C. and the Arthritis and Rheumatism Council. R.M.McM. is a Science Research Council CASE student.

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Chronic amphetamine administration and central dopamine receptor sensitivity

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Some psychotic disturbances in man may be associated with overstimulation of cerebral dopamine (DA) receptors and the chronic administration of amphetamine to animals may provide a model of these disorders (Snyder, 1973). Such administration potentiates stereotyped behaviour in rodents (Klawans, Margolin, Dana & Crossett, 1975) but it is difficult to explain why continued receptor stimulation should evoke an apparent hypersensitive response. We have therefore used the circling mouse, a model of dopamine receptor imbalance, to assess possible changes in DA receptor sensitivity during chronic (+)-amphetamine sulphate administration and following its withdrawal.

Male 'Swiss S' mice (20–25 g) showing consistent ipsiversive turning to (+)-amphetamine sulphate (4 mg/kg i.p.) and contraversive turning to apomorphine hydrochloride (0.25 mg/kg s.c.) two weeks following a 6-hydroxydopamine (16 µg in 4 µl 0.9% saline) induced lesion of one nigro-striatal pathway (von Voigtlander & Moore, 1973) were utilized. Animals were randomly divided into two groups ($n=30$). One group received increasing amounts of (+)-amphetamine sulphate in the drinking water until an approximate daily dosage of 20 mg/kg was reached after 1 month. This dosage level was continued for a further 2 months and then withdrawn. Control animals received drug-free drinking water during the experiment.

Chronic amphetamine administration was associated with an increase in spontaneous locomotor activity ($P<0.05$ at 2 and 3 months), as judged in Animex activity meters, although little (<1 turn/min) spontaneous circling behaviour was observed. Apomorphine-induced circling (0.01–0.5 mg/kg s.c.) when tested immediately prior to amphetamine administration and then at 1 week, 1, 2 and 3 months following drug administration was progressively reduced in comparison to control animals ($P<0.05$ at 2 and 3 months).

Spontaneous locomotor activity was decreased ($P<0.05$) up to 1 month following drug withdrawal and the circling response to apomorphine (0.01–0.5 mg/kg s.c.) remained depressed in comparison to control animals ($P<0.05$). Two months after drug withdrawal, spontaneous locomotor activity had returned to control values, although apomorphine-induced circling remained depressed.

Forebrain DA was decreased by 43% on the intact side after 2 months of chronic amphetamine administration and by 20% 1 month following drug withdrawal ($P<0.05$). The DA content of the lesioned side (27% of the intact side; $P<0.05$) was not further reduced by amphetamine administration.

Thus, chronic amphetamine administration inhibits apomorphine-induced circling, which might be due to increased stimulation of the intact striatal dopamine receptors. On the basis of this explanation the continued reduction in apomorphine-induced circling after amphetamine withdrawal would presume the development of supersensitivity of innervated striatal receptors. However, an alternative explanation might be that amphetamine administration decreases the response of nucleus accumbens to apomorphine and its withdrawal leaves accumbens receptors subsensitive to apomorphine.

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Transport of dopamine by rat blood platelets

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Blood platelets have been proposed as models for studying the uptake mechanisms in central aminergic neurones (Sneddon, 1973). Under conditions where initial rates of uptake are measured (i.e. using low substrate concentrations and short incubation times) the kinetics of platelet 5-hydroxytryptamine (5-HT) uptake and its pharmacological inhibition closely resemble the uptake of 5-HT by synaptosomes (Tuomisto, 1974). Human blood platelets also accumulate dopamine against a concentration gradient by a mechanism which is energy dependent and temperature sensitive (Boullin & O'Brien, 1970; Solomon, Spirt & Abrams, 1970). It is, however, unclear if this is a specific dopamine uptake mechanism and whether it has the same characteristics as the uptake mechanism in dopaminergic neurones in the corpus striatum.

Since most work on dopaminergic neurones has been carried out in rats (Snyder & Coyle, 1969; Horn, Coyle & Snyder, 1971) we have studied the uptake of dopamine by rat blood platelets to determine whether the platelet could be used as a model for the transport mechanism in dopaminergic neurones.

Uptake of dopamine by rat blood platelets was measured by a method similar to that described by Drummond & Gordon (1976) for uptake of 5-HT. Uptake of dopamine was linear for approximately 5 min at substrate concentrations of 1 μM or 20 μM. A biphasic curve was obtained when uptake was plotted against concentration (1 to 400 μM). This curve could be resolved into a saturable and a non-saturable component. The non-saturable component (which presumably represents diffusion) had a value of 40 pmol 10^8 cells^{-1} min^{-1} at 100 μM dopamine. Lineweaver-Burk analysis of the active uptake (i.e. after subtraction of the diffusion component) gave values of about 70 μM for the K_m and about 400 pmol 10^8 cells^{-1} 5 min^{-1} for V_{max}.

The active uptake of dopamine was abolished in the presence of an equimolar concentration of 5-HT but the diffusion was unaffected. Inhibition by 5-HT was not increased by preincubation, indicating that 5-HT exerted its inhibitory effect primarily at the platelet plasma membrane and not at the intracellular amine storage granule. Inhibition by low concentrations of 5-HT was decreased by preincubation; this is consistent with the rapid uptake of 5-HT by rat platelets (Gordon & Olverman, 1976). Kinetic analysis showed the inhibition to be competitive, with a K_i value of about 1 μM for 5-HT; this value is similar to the K_m for 5-HT uptake by rat platelets (Gordon & Olverman, 1976).

Chlorimipramine, desmethylimipramine and benztropine, which are respectively selective inhibitors of uptake into serotonergic, noradrenergic and dopaminergic neurones (Horn *et al.*, 1971; Horn, 1976) were tested for their ability to inhibit the uptake of dopamine and 5-HT by platelets. Substrate concentrations used were 0.8 μM for 5-HT and 20 μM for dopamine. Each drug was virtually equipotent against uptake of dopamine and 5-HT, although the absolute potency of the drugs varied greatly; IC_{50} values were about 0.1 μM for chlorimipramine, 3 μM for desmethylimipramine and 60 μM for benzotropine. Trenchard, Turner, Pare & Hills (1975) found that chlorimpramine had little effect on platelet dopamine uptake. However, in their study high substrate concentrations and long incubation times were used, and under these conditions diffusion (which is unaffected by chlorimipramine) would predominate.

We conclude that rat platelets do not have a specific active transport mechanism for dopamine, but that dopamine is transported into rat platelets via the 5-HT uptake mechanism, albeit with a much lower affinity. Consequently platelets cannot be regarded as models for studying the uptake of dopamine into central dopaminergic neurones.

This study was supported by a grant from the Arthritis & Rheumatism Council.

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Modification by anti-depressant drugs of plasma corticosterone levels in the stressed bulbectomized rat

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Rats following bilateral olfactory bulbectomy exhibit a motivational deficit in a one-way avoidance task (King and Cairncross, 1974), associated with a reduction in telencephalic noradrenaline concentrations. Amitriptyline (1.5 mg/kg i.p.) administered for 14 days prior to behavioural testing reversed the behavioural deficit (Cairncross, Schofield & King, 1973, Cairncross, King & Schofield, 1975). These observations suggested the bulbectomized rat as a screen for potential antidepressant drugs. The viability of such a premise has been confirmed using a variety of behavioural parameters (van Riezen, Schnieden & Wren, 1976, Rigter, van Riezen and Wren, 1977). This raised the question as to whether a physiological parameter could be used as an alternative indicant of antidepressant activity.

Bassett, Cairncross and King (1973) have established that plasma corticosterone elevation can be quantitated in the rat. Two degrees of corticosterone elevation were noted, an intermediate elevation (45–55 μg/100 ml blood plasma) and an extreme elevation (80–90 μg/100 ml blood plasma). Extreme glucocorticoid elevation occurred when a psychological stressor was superimposed on a physical stressor. Thus, it was decided to examine the effects of bilateral olfactory bulbectomy as a stressor and to expose bulbectomized and sham operated rats to predictable stress in the form of footshock (5 s of 1.5 mA scrambled footshock every 55 s for 20 min i.e. 20 trials).

It was demonstrated that footshock in sham operated rats produced intermediate 11-OHCS elevation, from a control value of 18.2 ± 1.6 to 44.4 ± 2.8 μg/100 ml blood plasma ($P > 0.001$). Bulbectomy without stress elevated plasma 11-OHCS levels to the intermediate level (43 μg/100 ml) and following regular footshock, the 11-OHCS level rose to extreme values (78.1 ± 3.04 μg/100 ml. $P > 0.001$). Similar patterns of elevation occurred in individually housed rats and in rats housed in groups of five.

The effects of antidepressant drugs were examined on the extreme steroid elevation induced by bulbectomy plus footshock. Amitriptyline and mianserin were administered at a dose of 5 and 10 mg/kg i.p. for 10 days and viloxazine at 2 and 5 mg/kg i.p. for 8 days to groups of bulbectomized and sham operated rats. The animals were subjected to footshock and immediately sacrificed. Whilst the three antidepressants had no effect on the intermediate 11-OHCS elevation induced by footshock in the sham operated groups, it was found that each drug significantly lowered the extreme steroid elevation in the bulbectomized groups.

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The ability of local injection of 6-OHDA, 5,6-DHT and 5,7-DHT into the olfactory bulbs, to mimic the effects of bilateral bulbectomy in the rat

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Bilateral removal of the olfactory bulbs produces a behavioural syndrome in rats which cannot be attributed to loss of olfactory sensibility (van Riezen, Schnieden & Wren, 1977). We have attempted to simulate bulbectomy by local injections into the olfactory bulbs of various neurotoxic drugs.

Male Wistar rats (150–220 g) were subjected to bilateral olfactory bulbectomy or sham-operation under anaesthesia. Fourteen days after surgery, the rats were tested for passive avoidance and irritability (van Riezen, Schnieden & Wren, 1977). On the day following the behavioural tests the rats were subjected to a regular footshock stress (Basset, Cairncross & King, 1973) and plasma samples collected for assay of 11-hydroxycorticosterone by the method of Mattingly (1962).

These experiments were repeated with rats which had received bilateral intrabulbar injections of 6-OHDA (5 and 10 μg/2 μl vehicle), 5,6-dihydroxytryptamine (5,6-DHT; 4 and 8 μg/2 μl vehicle) and 5,7-dihydroxytryptamine (5,7-DHT; 4 and 8 μg/2 μl vehicle). Another group received 5,7-DHT (8 μg) 30 min after an intraperitoneal injection of desmethylimipramine (25 mg/kg).

When compared with sham-operated rats, bulbectomized animals produced an increase in the number of trials required to acquire the avoidance response, an increase in the irritability score and an elevation of plasma 11-hydroxycorticosterone concentrations after stress (Table 1). 5,6-DHT (4 μg) produced a similar increase in irritability scoring only, whereas the high dose produced a syndrome identical to that seen after bulbectomy. 5,7-DHT (4 μg) also produced a significant increase in the irritability score. In contrast the high dose of this analogue brought about no changes in behaviour or steroid levels. In conjunction with desmethylimipramine pretreatment however, 5,7-DHT (8 μg) produced the full syndrome. After injection of 6-OHDA (5 μg), rats required significantly more trials to reach the learning criterion but no part of the syndrome was seen after injection of 10 μg 6-OHDA.

Cryostat sections of fixed brains were stained according to the Hjorth-Simonsen (1970) modification of the Fink-Heimer (1967) method for silver impregnation and observed for signs of neuronal degeneration under light microscopy.

Table 1 Effects of bulbectomy and drug injections on behaviour and plasma 11-hydroxy-corticosterone (11-OHCS) concentrations in rats[1]

Treatment	*n*	*Passive avoidance*[2] *(No. of trails)*	*Irritability score*[2]	*Stress-Induced [11-OHCS]*[2] *(μg/100 ml plasma)*
Bulbectomy	12	5.8 ± 0.3	6.8 ± 0.7	78.1 ± 3.04
Sham-operation	12	2.7 ± 0.4	1.7 ± 0.2	44.4 ± 3.04
Vehicle	6	3.6 ± 0.5	1.6 ± 0.4	42.1 ± 2.9
5,6-DHT 4 μg	9	3.8 ± 0.5	5.1 ± 0.4*	53.5 ± 2.2
5,6-DHT 8 μg	5	4.8 ± 0.4*	5.1 ± 0.8*	77.3 ± 4.3*
5,7-DHT 4 μg	3	3.0 ± 0.0	5.0 ± 1.0*	40.8 ± 7.6
5,7-DHT 8 μg	4	4.0 ± 0.4	2.0 ± 0.8	49.7 ± 5.7
5,7-DHT 8 μg + DMI (25 mg/kg)	6	5.8 ± 0.6*	4.5 ± 0.5	73.3 ± 5.0*
6-OHDA 5 μg	8	3.2 ± 1.0	2.2 ± 0.6	42.1 ± 2.04
6-OHDA 10 μg	10	4.0 ± 0.3	2.1 ± 0.3	44.4 ± 4.2

[1] Bulbectomy, sham-operation and bilateral injection into the olfactory bulbs (2 μl volumes of saline and ascorbic acid vehicle) were performed 14 days prior to testing.

[2] Results expressed as the mean and s.e. mean.

* Represents results which are not significantly different to those of bulbectomized rats.

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The xanthene-spiropiperidines: a new group of centrally-active drugs

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Any molecule which possesses a planar area (usually aromatic) and a nitrogen atom capable of occupying the same juxtaposition in space as the phenolic ring and the nitrogen of morphine can theoretically possess opiate properties. The Beckett and Casy model (Figure 1a) represents these requirements. From a variety of structures synthetized in a search for novel analgesic drugs, the xanthene-spiropiperidines of the general structure indicated in Figure 1(b) proved to be the most interesting. The similarity between the spiropiperidine moeity and morphine can be seen by comparison with Figure 1(c).

The unsubstituted spiropiperidine (ICI 81,058: R and R_2=H; R_1=Me) is virtually devoid of opiate properties (activity relative to normoprhine= <1% on the coaxially stimulated guinea pig ileum preparation [Paton (1957); Kosterlitz & Watt (1968)]), but is a potent sedative (54% decrease in locomotor activity of mice in photocell activity cages 1 h after 10 mg/kg p.o.) and antihistaminic (pA_2 v histamine on guinea pig ileum = 8.1). However, opiate properties are introduced when an hydroxyl is inserted at the 4 position (ICI 86,458: R=OH; R_1=Me; R_2=H). ICI 86, 458 has 16% of the potency of normorphine on the guinea pig ileum preparation and is analgesic (ED_{50} against acetic acid-induced writhing in mice = 1.3 mg/kg s.c.). The acetylated analogue (ICI 91,356: R=OAc; R_1=Me; R_2=H) is equipotent with normophine on the ileum and is a more effective analgesic (ED_{50} against acetic acid-induced writhing = 0.6 mg/kg s.c.).

Attempts to synthesize potent antagonists or partial agonists of the spiropiperidine series have met with little success e.g. the cyclopropylmethyl analogue (ICI 87,542: R=OH; $R_1=CH_2$-◁ ; R_2=H) has only 3% of the potency of naloxone on the mouse vas deferens preparation constantly perfused with etor-

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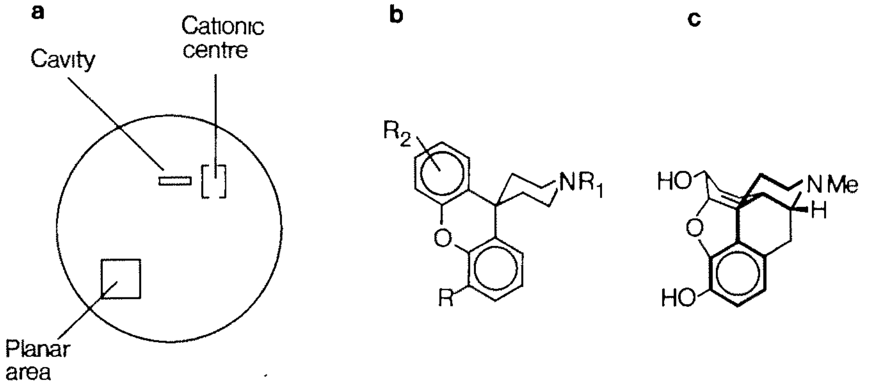

Figure 1 (a) Model of Beckett & Casy, 1954; (b) Spiropipendine; (c) Morphine.

phine (5 ng/ml).‡ It is speculated that this lack of antagonist activity may be due to the lack of rigidity of the piperidine ring.

Substitution in the 6 position in addition to the 4-OH substitution e.g. ICI 97,628 ($R=OH$; $R_1=Me$; $R_2=Cl$) leads to compounds with sedative and antihistaminic properties together with opiate activity. The pharmacological properties of ICI 97,628 have been investigated in considerable detail and will be described in this communication.

‡ We are grateful to Dr C. Smith of Reckitt and Colman Ltd., Dansom Lane, Hull, who advised us of this technique.

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A microiontophoretic study of enkephalin and enkephalin analogues on brain stem neurones in the rat

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Following the identification and synthesis of met- and leu-enkephalin and the demonstration of their opiate agonist activity in a variety of test situations, it became apparent that the intensity and duration of their actions was limited by rapid inactivation in brain tissue. Several peptides have now been synthesized which appear to be more stable. For example, substitution of D-alanine for glycine, in the 2-position of met-enkephalin results in a peptide which retains its affinity for opiate receptors, but which resists inactivation *in vitro* (Pert, Bowie, Fong & Chang, 1976). This analogue produces long-lasting analgesia in the rat when administered intra-cerebrally (Pert, 1976). A similar analogue of leu-enkephalin, Tyr-D-ala-Gly-Phe-D-leu (Burroughs-Wellcome BW 180C) has been shown to have analgesic activity when injected intra-cerebroventricularly (Baxter, Follenfant, Miller & Sethna, 1977).

With the alterations to the structure of the enkephalins, it was of interest to see what effects these novel peptides exerted at single neurone level, and also to compare their actions with those of the endogenous compounds. We have therefore investigated the effects of these compounds, applied microiontophoretically, on single medullary neurones, and compared the actions with those of leu- or met-enkephalin and etorphine. Male Sprague-Dawley rats were used, anaesthetized with urethane (1.2–1.8 g/kg) and prepared as previously described for recording from the medulla (Bradley & Dray, 1974).

Drugs used were D-(ala)^2-met enkephalin amide (17 mM pH 5.7), D-(ala)^2-D-leu^5 enkephalin-HCl (16.5 mM pH 3.0), met-enkephalin (15 mM pH 5.0), leu-enkephalin (15 mM pH 4.5), etorphine-HCl (25 mM pH 5.0), naloxone-HCl (25 mM pH 4.5). Ejection of Pontamine Sky Blue dye was used in many experiments to mark the position of cells responding to a peptide and/or etorphine.

Etorphine and the peptides consistently produced depression of the spontaneous activity of the cells to which they were applied. These depressions could be antagonized by prior or concurrent administration of the opiate antagonist naloxone. However, while the response to the enkephalins was of short duration, that to the stable analogues was much longer, lasting up to 10 min after the end of application. The depression of firing rate produced by etorphine was also long-lasting, as previously reported (Bradley, Briggs, Gayton & Lambert, 1976).

One striking feature of these experiments was the high percentage of cells affected by the peptides—some 80–90%. When morphine was tested in a similar manner (Bradley & Dray, 1974; Bramwell & Bradley, 1974) a lower percentage of cells responded and a significant number of naloxone-insensitive excitations were seen.

For the two enkephalin analogues tested, the amino-acid sequence modifications appear to confer greater stability without producing any qualitative change in the neuronal responses. This is entirely consitent with a transmitter role for enkephalin since it suggests that a mechanism exists for the rapid inactivation of the endogenous peptides *in vivo*.

LAL is an MRC Student.

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In vivo effects of GABA uptake inhibitors on the response of cat spinal neurones to GABA analogues

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The 'high' affinity uptake of GABA by slices of cat spinal cord is inhibited by relatively low concentrations of (+)-2,4-diaminobutyric acid (DABA), (−)-nipecotic acid, guvacine and arecaidine. The importance of such carrier-mediated uptake into neurones and glia in terminating the action of GABA *in vivo* has been suggested by the finding that these inhibitors enhance the inhibition of neurones by electrophoretic GABA in the cat central nervous system (Lodge, Johnston, Curtis & Brand, 1977). The possibility was not excluded, however, that the inhibitors enhanced the action of GABA by interactions at postsynaptic GABA receptors and/or ionophores.

Muscimol and isoguvacine are potent bicuculline-sensitive depressants of the firing of cat spinal neurones. In cat spinal tissue muscimol is a weak inhibitor of GABA uptake (33% inhibition at 2×10^{-4} M; GABA 10^{-8} M) whereas isoguvacine does not influence uptake significantly at 2×10^{-4} M. Thus high affinity GABA uptake processes seem unlikely to be involved in terminating the action of these two compounds *in vivo*, particularly that of isoguvacine.

Experiments were performed on 6 cats anaesthetized with pentobarbitone to compare the effects of the inhibitors of GABA uptake on the inhibition of spinal neurones by electrophoretic GABA, muscimol and isoguvacine. The action of muscimol was enhanced to a similar extent to that of GABA on all 10 cells tested by concentrations of (−)-nipecotic acid (7 cells), DABA (3 cells) and arecaidine (3 cells) which did not enhance the action of glycine.

The action of isoguvacine on 16 of 21 cells was not increased by the uptake inhibitors ejected in amounts which enhanced the inhibitory action of GABA: (+)-nipecotic acid (10 cells), DABA (5 cells), guvacine (15 cells). When the currents ejecting the inhibitors were increased 3–4 fold the action of isoguvacine was also increased. On the other 5 cells the inhibitors increased the effect of isoguvacine, but to a lesser extent than that of GABA, even when ejected with relatively low currents.

The results with muscimol indicate that the uptake inhibitors may interact cooperatively at GABA postsynaptic receptors/ionophores, so enhancing the effectiveness of GABA agonists, and/or interfere with transport processes which inactivate muscimol. The selectivity demonstrated using GABA and isoguvacine might indicate that low concentrations of the uptake inhibitors enhance the action of GABA by a relatively specific effect on cellular uptake. On the other hand this selectivity may have arisen from differences in the tissue distribution of electrophoretically administered GABA and isoguvacine. More direct evidence is required of the nature of possible transport processes for muscimol and isoguvacine in spinal tissue.

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The effects of noradrenaline and 5-hydroxytryptamine on the responses of dorsal horn neurones to noxious and innocuous skin stimuli

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There are numerous reports relating noradrenergic (NA) and tryptaminergic (5-HT) systems to the analgesia induced both by morphine and by stimulation in the midbrain (see Oliveras, Hosobuchi, Redjemi, Guilbaud & Besson, 1977, for references). Such an interaction could occur at the termination of descending NA or 5-HT fibres in the substantia gelatinosa of the spinal cord, a region in which morphine and methionine enkephalin amide have been shown to be active in reducing the nociceptive responses of neurones in laminae IV and V (Duggan, Hall & Headley, 1976, 1977). We have now tested NA and 5-HT administered electrophoretically in the dorsal horn.

Experiments were performed on 12 spinal cats anaesthetized with α-chloralose, paralysed with gallamine and artificially ventilated. The noxious stimulus to a hindlimb footpad was radiant heat (skin temperature > 45°C). Such a stimulus was alternated with an innocuous stimulus produced by deflection of nearby hairs by a moving airjet. Extracellular recordings were obtained with a multibarrel pipette positioned in spinal lamina IV or V, and drugs were ejected either from this pipette or from a second electrode positioned in the substantia gelatinosa, 50–760 μm dorsal and up to 100 μm lateral to the recording electrode. Results were obtained from 26 cells.

Administered in the substantia gelatinosa, NA (20–100 nA) and 5-HT (30–250 nA) reduced the nociceptive responses of 20/20 and 13/19 cells respectively. Selectivity (i.e. no reduction of responses to innocuous stimuli) occurred with 9 cells using NA and 6 cells using 5-HT; with most other neurones non-nociceptive responses were also reduced but to a lesser extent than were nociceptive responses. The time course of these effects was prolonged. Recovery from NA took 2–15 min and with 5-HT recovery took more than 20 min on 7 of 13 occasions. These actions contrast with the lack of effect of acetylcholine (150–200 nA, 7 cells), GABA (100–200 nA, 11 cells) and excitant amino acids (Duggan *et al.*, 1977).

Ejected near cell bodies 5-HT (22–100 nA) reduced both nociceptive and non-nociceptive responses of 4 of 6 cells tested and GABA (12–100 nA) was similarly non-selective on 7 of 10 cells. NA (10–40 nA) was slightly selective on 9 of 10 cells, but the degree of this selectivity was much less than with administration into the substantia gelatinosa.

Thus the monoamines, but not amino acids, were similar to morphine and enkephalin in being more selective for nociceptive responses when administered in the substantia gelatinosa. The opiate antagonist naloxone reduces the analgesia produced by stimulation in a nucleus of origin of descending 5-HT fibres (Oliveras *et al.*, 1977); we are currently testing naloxone for interaction with the monoamines administered in the substantia gelatinosa.

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Correlation between effects of brain-stem stimulation and effects of 5-hydroxytryptamine and noradrenaline on non-nociceptive and nociceptive spinal interneurones

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Electrical stimulation in the area of the brain stem raphé nuclei produces analgesia in the cat (Mayer & Liebeskind, 1974) which is thought to be produced by activation of a descending influence to cause inhibition of nociceptive impulses at the spinal level (Liebeskind, Guilbaud, Besson & Oliveras, 1973). It has been suggested that this descending pathway is tryptaminergic (Guilbaud, Besson, Oliveras & Liebeskind, 1973; Vogt, 1974). In order to investigate this possibility further, 5-hydroxytryptamine (5-HT) and noradrenaline (NA) were applied locally to identified dorsal horn interneurones and an attempt to correlate their effects on spontaneous and stimulus-evoked firing with effects of brain stem stimulation on their activity was made.

Cells were identified as having a nociceptive input if their activity was altered by injection of small amounts of bradykinin into the blood supply of their peripheral receptive field. Electrical stimulation in the area of the central interior mucleus of raphé generally caused inhibition of nociceptive cells but about half the cells classified as non-nociceptive were also inhibited to a lesser degree and for a shorter duration. On many cells, even when inhibition was seen, brain stimulation evoked one or two action potentials at short latency, prior to the inhibition, and there was no correlation with nociceptive input.

The effect of 5-HT on spontaneous activity was excitatory on both nociceptive and non-nociceptive neurones but it selectively inhibited activity evoked by noxious stimuli on nociceptive cells, indicating a presynaptic site of action. In contrast, NA generally had little effect on non-nociceptive cells whilst causing long-lasting inhibition of nociceptive cells and there was a correlation between the amount of inhibition (measured as the percentage inhibition of control firing) caused by NA and the percentage inhibition from brain stem stimulation.

From these results it would appear that 5-HT is unlikely to be a candidate as the transmitter mediating inhibition from brain stem stimulation. However, descending excitatory tryptaminergic pathways may increase activity of interneurones which in turn inhibit other neurones transmitting nociceptive impulses.

G.B. is an MRC Student. The research was supported by a grant from the Welcome Trust.

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On the structural requirements for dopamine-like activity in homogenates of rat nucleus accumbens

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The dopamine-sensitive adenylate cyclase present in homgenates of rat striatum (Kebabian, Petzhold & Greengard, 1972) has proved to be a useful model system for the study of drugs affecting mammalian dopamine receptors (review by Iversen, 1975). Dopamine sensitive adenylate cyclases are also present in other brain regions including the nucleus accumbens (Horn, Cuello & Miller, 1974). We have now investigated the structural requirements for dopamine-like activity on the adenylate cyclase from rat nucleus accumbens.

The activity of adenylate cyclase in homogenates of rat nucleus accumbens was estimated using the method of Kebabian, Petzhold & Greengard (1972) and the resulting cyclic AMP was determined according to Gilman (1970). Potency of agonists was expressed as a percentage of the maximum response which was taken as that produced by 100 μM dopamine. A log concentration/response curve was constructed for each compound and EC_{50} values (concentration producing 50% of maximum response) were obtained.

In the absence of dopamine, the mean production of cyclic AMP by 50 μl nucleus accumbens homogenates was 49.9 ± 9.4 ($n = 12$) pmol/tube during the 3 min incubation. This was increased to 80.2 ± 9.4 ($n = 12$) pmol/tube by 100 μM dopamine. The potency of the compounds tested is shown in Table 1.

Table 1 Potency of drugs in increasing cyclic AMP production in homogenates of rat nucleus accumbens

Agonist	*EC_{50} (μM)*
ADTN*	0.7 ± 0.3
Epinine	1.8 ± 0.8
Dopamine	6.3 ± 1.2
(—)-Noradrenaline	25.1 ± 14.6
(—)-Adrenaline	50.1 ± 17.2
3,4-dihydroxy-5-methoxy phenylethylamine	141.3 ± 17.2
(±)-Isoprenaline	Inactive
(—)-Phenylephrine	Inactive
3,5-dihydroxy-4-methoxy phenylethylamine	Inactive
3-hydroxy-4,5-dimethoxy phenylethylamine	inactive

* 2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene.
EC_{50} values are the mean ± s.e. mean obtained from 4–8 observations.
All active compounds gave 100% stimulation.

The order of potency of the various agonists is similar to that previously determined in homogenates of rat striatum (Miller, Horn, Iversen & Pinder, 1974; Munday, Poat & Woodruff, 1976), suggesting a similarity between the dopamine receptors in the two regions of the brain. The high potency of ADTN is consistent with previous behavioural and electrophysiological studies with this compound.

K.J.W. is an MRC student.

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The effects of some indolalkylamines on the uptake and release of 5-hydroxytryptamine in rat striatum

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The mammalian CNS shows a differential anatomical distribution of 5-hydroxytryptamine (5-HT) (Bogdanski, Weissbach & Undenfriend, 1957) and *in vitro* exhibits an active transport system for the uptake of 5-HT which is distinguishable from that of noradrenaline and dopamine (Blackburn, French & Merrills, 1967; Shashkan & Snyder, 1970). In this study we have investigated the effects of various indolakylamines on the uptake and release of [^{3}H]-5-HT *in vitro* providing complementary data to the structure-activity study of Horn (1973) with these compounds on the uptake of 5-HT.

The method of Raiteri, Angeli & Levi (1974) was used; briefly rat striatum tissue was removed, weighed and chopped into cubes 0.1 × 0.1 × approximately 2.0 mm. The tissue was suspended in 10 ml cold incubation medium (containing 5.10^{-5} M pargyline) and pre-incubated for 15 min at 37° and subsequently incubated for 10 min [^{3}H]-5-HT at a final concentration of 1.10^{-8} M (5-[1,2-^{3}H(N)]; S.A. 22.5 Ci/mmol). In uptake experiments the drug was added simultaneously with the radioactive 5-HT and after the incubation the tissue was separated from its incubation medium either on Millipore filters or by centrifugation at 10,000 × g. The tissue, or tissue and filter, was solubilised with PCS (Amersham-Searle) and the amount of radioactivity accumulated determined by liquid scintillation counting. The results were corrected for non-specific uptake by subtraction of a zero time blank. In the release experiments the radiolabelled tissue resting on Millipore filters was superfused with incubation medium at 37°. This was drawn through the tissue bed at 0.5 ml/min and fractions were collected every minute. After 5 min the superfusing medium was replaced by a medium containing the indolakylamine studied and superfusion continued for a further 10 minutes. The percentage of total recovered radioactivity (filter + tissue + fractions) was calculated for each fraction and the combined values for the first 5 fractions subsequent to the initiation of release corrected for basal release, this value was expressed as a percentage of the maximal release of [^{3}H]-5-HT caused by cold 5-HT (1.10^{-4} M).

IC$_{50}$ values were calculated from semi-log plots and in this preparation were found to be $2.6 \cdot 10^{-7}$ M for tryptamine, $2.7 \cdot 10^{-7}$ M for α-methyltryptamine, $5.5 \cdot 10^{-6}$ M for 5-methoxytryptamine and $1.9 \cdot 10^{-8}$ M for chlorimipramine. In the release experiments chlorimipramine (10^{-5} M) caused no release of 5-HT while tryptamine, α-methyltryptamine and 5-methoxytryptamine at the same concentration caused 69%, 71% and 41% release respectively calculated as described above. At a concentration of 10^{-7} M the values for tryptamine, α-methyltryptamine and 5-methoxytryptamine were 36%, 42% and 0% respectively.

In comparison with chlorimipramine, tryptamine and α-methyltryptamine were approximately an order of magnitude less potent as inhibitors of 5-HT uptake, while 5-methoxytryptamine was still less potent. However while chlorimipramine exhibited no ability to release 5-HT, the other indolakylamines investigated showed the same rank order of potency on release as against uptake inhibition.

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Effects of chronic (±)-propranolol on catecholamines and GABA in rat striatum

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Investigations of the effects of (±)-propranolol on central catecholamine concentrations have produced conflicting results (Peters & Mazurkiewicz-Kwilecki, 1975). However we have shown that chronic administration of propranolol to rats increased steady state concentrations of dopamine in the corpus striatum. (Mahon, O'Donnell & Leonard, 1977). In the study reported here we have attempted to extend our previous findings.

Male Sprague-Dawley rats (180–200 g) were given (±)-propranolol (30 mg/kg) daily for 14 consecutive days in a divided dose. Diazepam (5 mg/kg) was similarly administered to another group and controls received saline. All injections were given i.p. Behaviour was assessed on day 12 using a modified form of the hole-board of Davies & Wallace (1976). Twelve h after the final injections the concentrations of γ-amino-n-butyric acid (GABA), noradrenaline (NA) and dopamine (DA) in the midbrain and striatum were estimated following microwave radiation of the skulls. The catecholamines were determined by the method of Leonard & Tonge (1969) and GABA by the method of Uchida & O'Brien (1964). Striatal catecholamine turnover following similar propranolol treatment was measured in a second experiment using the MAO inhibitor, pargyline.

Chronic administration of propranolol caused a significant ($P < 0.005$) decrease in striatal GABA concentration and increases ($P < 0.05$) in striatal DA and midbrain NA levels. Neither chronic diazepam nor an acute dose of propranolol (30 mg/kg) had any effect on the neurotransmitters measured. No effects of propranolol on turnover were detected. The animals' behaviour was unaltered by any of the drug treatments.

An increase in steady state DA concentration in the striatum could be due to direct receptor blockade, and/or to an effect on tyrosine hydroxylase activity in this brain area. Propranolol has been shown to differ from the phenothiazines in that it augments the rotation produced by amphetamine (Fuxe, Bolme, Agnati & Everitt, 1976). Peters & Mazurkiewicz-Kwilecki (1975) have reported a stimulation of striatal tyrosine hydroxylase activity in rats given propranolol for 6 days. Our results are compatible with an action of the drug on this enzyme.

This work was supported by a grant from Organon, B.V. (Netherlands). Propranolol and Pargyline were gifts of I.C.I. Ltd. and Abbot Laboratories respectively.

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Reactions of morphine-dependent rats to changes in their usual daily dose

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Rats that are maintained on large, once daily doses of morphine, eat, drink and are active for up to 6 h after injection. During the next 18 h they eat and drink less, defecate more, lose weight and become less active (Kumar, Mitchell & Stolerman, 1971); these are all signs of morphine abstinence (Martin, Wikler, Eades & Pescor, 1963). We have tested how such rats respond to abrupt changes in the size of their usual daily dose of morphine. Male hooded rats ($n = 12$) were injected intraperitoneally with increasing doses of morphine-HCl once a day at 10 h and were then maintained on 100 mg/kg for 48 days. Then, instead of the usual daily dose, test doses of morphine 0, 25, 50 and 100 mg/kg were given in counterbalanced order at weekly intervals and measurements of food and water consumption were made at intervals during the next 24 h; defecation was measured by the number of boluses deposited and locomotion was tested during a 3 min test in a Y-maze 2.5 h after the dose. Between tests rats received the usual daily dose of morphine. Analyses of variance were performed on the scores and assessments of linear trend tested for dose-related changes. Over the 24 h there were dose-related falls in eating and drinking, increases in defecation and in weight loss; locomotion was also reduced ($P < 0.001$ in all cases). Without morphine the signs of abstinence were most intense and occurred mainly during the first 6 h after injection.

In the second experiment, male hooded rats ($n = 12$) were maintained on 100 mg/kg of morphine for 78 days and were tested using the same general procedure as above. However, the weekly test doses in this experiment were increased from 100 mg/kg to 125, 150 and 200 mg/kg of morphine. The main consequence was a dose-related reduction in the intensity of abstinence signs between 6–24 h after injection. The nightly falls in eating and drinking, the defecation and weight loss were all mitigated (linear trend significances: $P < 0.01$). There were depressant effects on eating and defecation of the raised doses during the first 6 h after injection but drinking and locomotion were unaffected. On the next day, however, 6–24 h after the usual daily dose of morphine, there were some increased signs of abstinence as a function of the previous day's test dose—the rats drank less ($P < 0.001$), defecated more ($P < 0.05$) and lost more weight ($P < 0.001$); for example, after 200 mg/kg, mean fluid consumption fell from 16.0 ml to 7.8 ml, defecation increased by 21% and weight loss rose from 11.1 g to 22.7 g. Therefore, interpolating even a single large dose can temporarily alter the level of morphine dependence.

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Effect of vinblastine sulphate on the acquisition of a conditioned avoidance response in the goldfish

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The alkaloid colchicine is known to disrupt neurotubules (Banks & Till, 1975). It has been suggested that neurotubular integrity is necessary during learning since intracranial (i.c.) injections of colchicine in goldfish (*Carassius auratus*) interfere with the acquisition of an active shock avoidance learning paradigm (Clingbine & Heading, 1977). Other drugs which disrupt neurotubule integrity e.g. the *Vinca* alkaloid vinblastine sulphate (VLB) (Wooten, Kopin & Axelrod, 1975) may cause similar behavioural effects.

Goldfish (6–8 g) were injected with vinblastine (1 μg, i.c.) while anaesthetized with MS222 (tricaine-methane-sulphonate, Sandoz) at a concentration of 200 μg/ml. They were allowed to recover for 6 h in their home tanks prior to initial training. They were then trained at 18°C in a two-way shuttlebox to associate a light (CS) with an electric shock (US) by being given 20 trials per day over 5 days. Performance was judged by the number of responses to the CS and analysed by ranking tests (values of $P < 0.05$ were taken as significant). After 5 days, the performance of fish injected with vinblastine (1 μg) was significantly lower than controls receiving vehicle (10 μl of Young's Freshwater Teleost Ringer solution). Controls significantly improved over 5 days but vinblastine injected fish did not.

Other fish were tested to see if unconditioned behaviour was affected by the drug. This was assessed by two methods *viz.* the number of times that free-swimming fish spontaneously crossed between compartments of the shuttlebox and the time taken to escape from the US. No significant difference was observed in the incidence of spontaneous crossing between chambers of the shuttle box or in the time to escape from the US in controls compared to vinblastine injected fish. The usefulness of spontaneous crossing and shock escape time in detecting behavioural changes was shown in fish immersed in MS222. At a concentration of 20 μg/ml of anaesthetic, fish made significantly fewer spontaneous crossings, and at 33 μg/ml took significantly longer to escape from the US, compared to fish in water alone.

In order to determine whether the effect of vinblastine on avoidances was dose related, other fish were given either 0.5 μg or 2 μg of vinblastine (i.c.). After 5 days, the performance of the two groups was not significantly different but both groups avoided shock significantly less well than controls.

Vinblastine is known to disrupt the integrity of neurotubules, probably by combining with a subunit protein (Itani & Lagnado, 1976). It is speculated that the observed behavioural effects are due to disruption of either neurotubule structure or function, or possibly both. The results obtained are unlikely to be due to general toxic actions since vinblastine injected fish escaped from shock as well as controls and were also just as spontaneously active.

G.C. is in receipt of a Science Research Council Studentship.

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DEMONSTRATIONS

A modular system of instruments for simple experiments on skeletal muscle using intracellular microelectrode techniques

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The apparatus to be demonstrated forms a basic system used routinely in three laboratories over the last five years. The system consists of a buffer amplifier, constant current generator, current monitor and differentiator. The circuits are essentially derived from those described by Clayton (1971).

The buffer amplifier employs a 748 operational amplifier with a dual field effect transistor (F.E.T.) source follower at the front end. It features input capacitance neutralisation and a facility for checking electrode resistance. The input impedance of the amplifier is of the order of 10^{12} ohms and a typical rise time of 60 µs is obtained when used with a 10 MΩ electrode connected to the amplifier via a 25 cm long low noise input lead.

The constant current generator consists of a high gain amplifier with a dual F.E.T. front end with two input and two feedback resistors connected to form a constant current generator. Additional transistor switching circuitry is employed to drive the generator at the required trigger voltages, enabling it to produce both steady and pulsed currents of up to ± 200 nA.

The timing of the pulse current is obtained by triggering the generator from two output sockets of a Digitimer Constant Voltage Generator.

A capacitance neutralization amplifier similar to the buffer amplifier is also built-in enabling the user to square up the stimulating current pulse and to monitor the condition of the microelectrode. An electrode resistance measuring facility is also employed.

The current monitor is also a high gain amplifier with a dual F.E.T. front end with a single feedback resistor. Connected this way the circuit forms what is commonly known as a current to voltage converter, producing a voltage output directly proportional to the current being monitored.

The differentiator circuit is typical of 'active' differentiating circuits for measuring the rate of rise of action potentials and is meant to be driven from the low output impedance socket of the buffer amplifier. It features an input impedance of 1 MΩ and will give an output of 1 V maximum for rates of rise of either 0–1000 V/sec or 0–10 V/seconds.

The apparatus is constructed as a modular system allowing considerable flexibility in use. It is easily assembled and has a total component cost of approximately £250. A full description of the circuit is available.

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A modified guinea-pig stomach preparation

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Several isolated preparations have been used to investigate the motility of the guinea-pig stomach (for references see Beani, Bianchi & Crema, 1971) but all these preparations were relatively insensitive to relaxant drugs and the relaxations were slow in onset. In the isolated preparation demonstrated, these problems have been overcome by injecting drugs into the vasculature of the stomach, via the coeliac axis.

Female guinea-pigs (250–400 g) were starved for 24 h before being killed by cervical dislocation. The vagal nerves were located and dissected clear of the oesophagus, which was ligated. The coeliac axis was cannulated before the stomach was removed from the animal; the spleen was ligated and removed, ensuring that the blood supply to the greater curvature of the stomach was intact. A bulk ligature was tied around the fat surrounding the hepatic artery and the pancreatic and duodenal artery was ligated. Freshly oxygenated McEwen's solution (4 ml) was slowly injected through the vasculature. The stomach, with the cannula attached, was then removed from the animal and a hole cut in the fundus to wash the lumen clear of chyme. The tube of a modified Trendelenburg apparatus was tied into the fundus, the pylorus was ligated and the preparation set up in a 100 ml isolated organ bath filled with McEwen's solution maintained at 35 ± 1°C, gassed with 95% O_2: 5% CO_2. The tube of the apparatus was connected to a reservoir of McEwen's solution; the level of fluid in the reservoir was initially set 3 cm above the fluid level in the organ bath. Air pressure over the reservoir was measured using an Ether UPI air pressure transducer and a Devices M2 recorder.

Stimulation of the vagal nerves resulted in a contraction of the stomach and a displacement of McEwen's solution from the lumen of the stomach into the reservoir. Hyoscine (0.3 µM) converted the contraction into a relaxation; the increase in volume of the stomach following vagal stimulation (5 Hz for 10 s) was 2.0 ± 0.4% (s.e. mean, $n = 13$; resting volume 29 ± 6 ml). In the presence of hyoscine, relaxations were consistent for 30 hours.

Drugs, dissolved in 0.1 ml McEwen's solution, were injected via the coeliac axis and followed by 0.4 ml McEwen's solution. Relaxations induced by (−)-noradrenaline (0.1–10 nmol) and ATP (0.1–10 µmol) were rapid in onset and produced a maximum effect within 10 seconds. The relaxations resembled the responses to vagal stimulation the presence of hyoscine. The drug-induced relaxations were not tachyphylactic and were not antagonized by tetrodotoxin (0.3 µM). The preparation was more than 80 fold more sensitive to noradrenaline and more than 30 fold more sensitive to ATP when the drugs were injected via the coeliac axis than when added to the McEwen's solution in the organ bath.

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Regional perfusion of the airways of guinea-pig lung: a selective action of histamine on the smooth muscle of the small airways

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The direct actions of drugs on the smooth muscle of lung have usually been investigated using muscle of the larger airways (Castillo & de Beer, 1947; Coleman & Farmer, 1970) despite evidence from experiments *in vivo* that the principal site of action is the fine peripheral airways (Drazen & Austen, 1974). The actions of histamine and acetylcholine on the perfused airways of guinea-pig lungs have been investigated using modifications of the preparation of Sollman & Von Oettingen (1928).

Lungs were removed from guinea-pigs (350–650 g) and washed free of blood with Krebs solution (NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; MgCl 1.2; $NaHCO_3$, 25.0; NaH_2PO_4 1.2; glucose 11.5 mM) via a cannula in the pulmonary artery. Whole lungs were perfused via a polythene cannula tied into the trachea, the tip lying about one-third of the distance from the larynx to the bifurcation of the major bronchi. The fluid escaped from fine scarifications on the surface of the lobes. Half lungs were cannulated through the lower trachea and the cannula tied into the bronchus so that the tip lay 2–4 mm beyond the bifurcation. Single lobes were perfused via a fine cannula introduced through the bonchus such that the tip lay in the centre of the lobe; all other lobes were tied off and removed. Preparations were suspended in a constant temperature chamber and perfused with Krebs solution gassed with 5% CO_2 in O_2 at 5, 2.5 and 2 ml/min respectively. Perfusion pressure was measured by a transducer attached to a side arm above the cannula. Agonists were injected into the perfusion fluid in volumes of 0.1 ml through a thick-walled elastic tubing between the side-arm and the cannula.

Acetylcholine (100 ng–200 µg) and histamine (10 ng–100 µg) caused dose-dependent increases in perfusion pressure in all preparations. The order of sensitivity to both agonists was left lower lobe > right lower lobe and upper lobes ≥ half lungs > whole lungs (ED_{50} for histamine = 25:141:141:4000 ng respectively and for acetylcholine = 0.63:3:14:18 µg respectively). Also the maximum pressure attained with left lower lobes (mean ± s.e. = 155 ± 18 mm Hg; $n = 14$) was greater than that attained with other preparations (66 ± 10 mmHg; $n = 20$).

The greater sensitivity of perfused lobes compared with whole lungs to histamine (ratio $ED_{50}s = 16$) against acetylcholine (ratio $ED_{50}s = 2.9$) suggests that histamine has a relatively greater effect on smaller airways. In addition, the perfused single lobe provides a very sensitive simple preparation for the study of drugs on the smooth muscle of small airways.

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The use of rat atria as a simple and sensitive *in vitro* preparation for detecting pre-synaptic actions of drugs on adrenergic transmission

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Several workers have used the inotropic and chronotropic responses of mammalian heart to investigate the actions of drugs on the release of adrenergic transmitter. Sympathetic cardiac nerves have been electrically stimulated in pithed rats thereby producing tachycardia and the ability of drugs to modify this tachycardia in various ways has been examined (Armstrong & Boura, 1973; Drew, 1976). The positive inotropic response of guinea-pig atria due to electrical stimulation has been utilized by Vizi, Somogyi, Hadhazy & Knoll (1973) to investigate the role of α-adrenoceptors in adrenergic transmission.

The present demonstration shows a very simple and sensitive preparation *in vitro* which can be successfully used to monitor the actions of drugs on adrenergic transmitter release.

After killing a male albino rat by concussion, the heart is taken out and transferred to Krebs-Henseleit solution at room temperature. The atria are dissected free and set up in a 10 ml organ bath between parallel platinum electrodes in Krebs solution containing atropine sulphate (5 μM) at 37°C, and bubbled with 95% O_2, 5% CO_2 mixture. The atrial beats are recorded through an isometric transducer on a Devices Recorder. Each period of electrical field stimulation comprises of three trains, each of five pulses (0.5 ms duration, 10 Hz, 12 V) delivered at 30 s intervals. Atrial beats are counted for 10 s and are expressed as beats/minute.

Electrical field stimulation always increases the atrial rate, e.g. in 7 experiments, the increase has been: first train 34.1 ± 4.0; second train 42.1 ± 6.7; third train 46.4 ± 7.4 beats/min (mean ± s.e. mean). The increase in rate following stimulation is reversible and the atria revert back to their pre-stimulation rate within 2–5 minutes. The following observations strongly suggest that the increase in atrial rate after electrical stimulation is due to the release of noradrenaline from adrenergic nerves.

(1) Propranolol, 30 nM, abolishes the increase in auricular rate following electrical stimulation.
(2) Clonidine, 10 nM, known to possess adrenergic neurone-blocking property (Idowu & Zar, 1976), inhibits the increase in the auricular rate following field stimulation (% inhibition ± s.e. mean: 55.17 ± 9.25). This action of clonidine is lost after treatment with phentolamine (5 μM). Phentolamine itself does not exert any significant effect on the auricular rate.

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Effect of vinblastine and mustine on the distribution of T and B-lymphocytes

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Differential prostaglandin production by microsomal fractions of rat pregnant uterus

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Crude homogenates of decidual tissue from rat pregnant uteri synthesize prostaglandin (PG) F as estimated by parallel bioassay (Williams, Sneddon & Harney, 1974). As recent work has shown the increasing complexity of arachidonic acid metabolism we have developed a radiometric assay to allow more detailed analysis of products formed by the uterus.

Pregnant rats were killed (day 22 of pregnancy) and 20% homogenates of deciduum and myometrium prepared in Tris/acetate buffer (pH 8) containing hydroquinone 0.5 mM and reduced glutathione 2 mM. Microsomes were prepared by ultracentrifugation and samples incubated with 1 ml aliquots of buffer containing arachidonic acid (99 nmol) and [^{14}C]-arachidonic acid (3.5 nmol) for 30 min at 37°C. Pilot experiments indicated these conditions were optimal for decidual tissue with regard to substrate and co-factor concentrations, pH and incubation time. Samples boiled before incubation served as controls. After solvent extraction residues were subjected to thin-layer chromatography (TLC) using the solvent chloroform 90:methanol 9:acetic acid 1:water 0.65 by volume. The radioactivity in consecutive 0.5 cm bands of each zone was then estimated by liquid scintillation counting. Authentic $PGF_{2\alpha}$, PGE_2, [^{14}C]-6-oxo $PGF_{1\alpha}$ (prepared as described by Cottee, Flower, Moncada, Salmon & Vane, 1977) and arachidonic acid were used as markers.

With decidual microsomes 23% of radioactivity on the TLC plate was converted to products other than monohydroxy acids (range 14–50%, 15 experiments). 50% of this radioactivity had an R_F value similar to PGE_2, 25% ran as PGD_2 while 20% had a mobility equivalent to $PGF_{2\alpha}$. Myometrial substrate conversion was lower, 6% (range 2–15%, 15 experiments) and all the radioactivity was located in the PGE_2 zone. However, in this solvent system PGE_2 and 6-oxo $PGF_{1\alpha}$ have similar R_F values. Therefore the zones of radioactivity corresponding to PGE_2 were removed, eluted and rechromatographed using solvent systems devised to separate these two substances (Cottee *et al.*, 1977). Using these TLC systems we found that approximately 12% of radioactivity in the decidual PGE zone behaved as 6-oxo $PGF_{1\alpha}$ and 70% as PGE_2. However 84% of myometrial radioactivity appeared to be 6-oxo $PGF_{1\alpha}$ and only 6% PGE_2.

Thus decidual microsomes produce PGE_2 as the major product whereas myometrial preparations synthesize predominantly 6-oxo $PGF_{1\alpha}$. Further investigation is needed to determine whether this myometrial PG production plays any part in regulating uterine function during pregnancy.

We thank the Wellcome Trust for a grant.

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Accumulation of platelets at acute inflammatory sites

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The involvement of blood platelets in acute inflammatory reactions is a topic of some interest. One experimental approach has been the use of animals made thrombocytopenic by treatment with anti-platelet sera. Some workers have reported that such animals either show diminished responsiveness to reactions such as the active Arthus and Shwartzman (Margaretten & McKay, 1969, 1971) or a decreased appearance of platelet-like cells into developing inflammatory exudates (Smith, Walker, Ford-Hutchinson & Penington, 1976). In other animal models of acute inflammation, such as hind paw oedemas, it has been reported that platelet-like bodies occur in the swollen paw (Rédei & Kelemen, 1969) but that the development of the oedema is not affected by pre-treatment of the animals with anti-platelet sera (Ubatuba, Harvey & Ferreira, 1975).

We have examined the distribution of platelets and erythrocytes in and around implanted inert sponges in the rat. Female albino Wistar rats (150–200 g) received either homologous platelets labelled with ^{51}Cr (sodium chromate) according to the method of Radegran (1976) or homologous erythrocytes labelled with ^{51}Cr according to the method of Gray & Sterling (1950). Eighteen hours after labelling, the animals were subjected to an inflammatory insult, either subdermal implantation of 3 to 6 saline soaked sponges or subdermal incisions without sponge implantation. The rats were killed after various time intervals and the sponges, the skin around the sponges or incision, skin from other areas and blood samples were removed for determination of radioactive content.

The results of the experiments showed that the labelled platelets appeared only in the sponge exudates to a minor degree but that they preferentially accumulated in the vascular bed around the implanted sponges and in the other areas of skin examined. It is suggested that if platelets have a role in the early phases of the development of inflammatory reactions then this is intravascular rather than extravascular.

Supported by the Wates Foundation.

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Estimation of plasma nicotine by combined capillary column gas chromatography–mass spectrometry

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A quantitative method for the determination of nicotine by combined capillary column gas chromatography–mass spectrometry has been developed. The initial extraction of nicotine, from plasma, used a modification of the method of Feyerabend, Levitt & Russell (1975). This modification allowed the addition of the quinoline internal standard direct to the plasma, prior to extraction.

The nicotine was finally extracted into benzene and injected (0.8 µl) into a gas chromatograph fitted with a 20 m (0.3 mm internal diameter) glass capillary column coated with SP1000. The initial column temperature was 140°C and this was increased at 6°C/min to a final temperature of 180°C.

A V.G. Micromass 12B2 mass spectrometer was directly coupled via a 0.15 mm internal diameter glass capillary restriction to the gas chromatograph. The mass spectrometer operating conditions were: interface 250°C, ion source 200°C, ionizing potential 70 eV, accelerating voltage 4 KV, and source pressure 10^{-5} mmHg.

The most abundant ion in the nicotine spectrum (mass to charge ratio (m/e) 84) and the molecular ion of quinoline (m/e 129) were measured by selective ion monitoring. The accelerating voltage was switched between 2.6 and 4 kV to bring these ions into focus.

The ratio of the m/e 84 and m/e 129 peak heights was linearly related to nicotine concentration over the range 5–100 ng nicotine/ml plasma.

This method has been applied to the study of the pharmacokinetics of nicotine in man and experimental animals.

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Br. J. Pharmac. (1977), **61**, 163–166

EFFECT OF INTRAHYPOTHALAMIC PHENTOLAMINE ON HYPOTHERMIA PRODUCED BY PERIPHERAL NORADRENALINE IN THE PIGEON

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1 The effect of intrahypothalamic phentolamine (1 and 2 µl of a 10 µg/µl solution) on hypothermia induced by intramuscular injection of noradrenaline (NA, 2 mg/kg) at different times (2, 10, 20 min) after phentolamine was investigated in pigeons.

2 Administration of phentolamine shortly before NA was shown to attenuate hypothermic responses to NA.

3 It is suggested that the attenuation is due to blockade of central hypothermic effects of intramuscular NA.

4 The mapping of injection sites in the brains was carried out.

Introduction

Although catecholamines including noradrenaline (NA) are generally believed to be incapable of penetrating the blood-brain barrier of adult animals in more than minute amounts (Whitby, Axelrod & Weil-Malherbe, 1961; Oldendorf, 1971; 1974), these amines, even when peripherally applied, may have central thermoregulatory effects (Allen & Marley, 1967; Feldberg & Lotti, 1967). In birds, peripheral (Allen & Marley, 1967; Allen, Garg & Marley, 1969; 1970; Hissa, Pyörnilä, & Saarela, 1975a; Hissa, Saarela & Pyornilä, 1975c), intracerebroventricular (Grunden & Marley, 1970; Chawla, Johri, Saxena & Singhal, 1974) and intrahypothalamic (Marley & Stephenson, 1968; 1969; 1975; Hissa & Rautenberg, 1974; 1975) injections of catecholamines at or below thermoneutrality have been shown to induce hypothermia. Chawla *et al.* (1974) and Hissa & Rautenberg (1975) demonstrated that the hypothermic effect of centrally injected NA was diminished or totally prevented by an intracerebral injection of phenoxybenzamine or phentolamine. Based on these findings it seemed logical to ask whether an intrahypothalamic injection of an α-adrenoceptor blocking agent might also affect the hypothermia induced by a peripheral injection of NA. This has been tested in conscious pigeons.

Methods

Adult pigeons (*Columba livia*) of either sex (weight 300–350 g) were used. The birds were trapped at Turku, SW-Finland in October-November, and the experiments carried out in January-June. The care of birds has been described earlier (Pyörnilä, Hissa & Saarela, 1976).

Stereotactic implantation of a guide cannula into the hypothalamus was performed as described by Hissa & Rautenberg (1974). The birds were allowed a week for postoperative recovery and were then tested with an intrahypothalamic injection of NA (10 µg/µl) at an ambient temperature (*t*a) of 6°C. Pigeons that responded with hypothermia (see Hissa & Rautenberg, 1974) were used further. In experiments at *t*a 6°C, phentolamine (10 µg/µl or 20 µg/2 µl) was injected into the hypothalamus followed by NA (2 mg/kg) intramuscularly at different times (2, 10, 20 min) after phentolamine.

The method for measuring O_2 consumption (Beckman E2 Analyzer) was that described by Hissa & Palokangas (1970). Also previously described were the techniques for intrahypothalamic and peripheral injections (Hissa & Rautenberg, 1974; Hissa *et al.*, 1975c; Pyörnilä *et al.*, 1976) and the methods for measuring body (*t*b) and foot temperatures (*t*f) (Ellab Z8 recorder) and shivering (breast muscle electrodes and Schwarzer Varioscript V822 polygraph, see Saarela, Mattila, Hissa & Hohtola, 1976). At the end of the experiments the birds were killed and the brains fixed in 10% formalin. Paraffin sections (15–20 µm) were stained with cresylviolet and examined for the location of the injection cannula.

An experiment was not begun until stable baselines for *t*b and O_2 consumption were obtained (usually within 2 hours). In each experiment the mean *t*b and

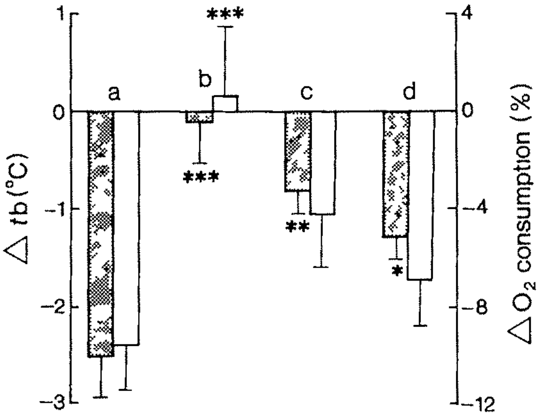

Figure 1 Mean maximum change in body temperature (Δ*tb*, stippled columns) and O_2 consumption (open columns) in response to noradrenaline (NA, 2 mg/kg, i.m.) alone and after preinjection of phentolamine into the hypothalamus. (a) NA without phentolamine (10 experiments on 7 pigeons); (b) NA 2 min after phentolamine, 20 µg/2 µl (16 experiments on 7 pigeons); (c) NA 10 min after phentolamine, 20 µg/2 µl (13 experiments on 6 pigeons); (d) NA 20 min after phentolamine, 10 µg/µl (11 experiments on 6 pigeons). Vertical bars represent s.e. means. Asterisks refer to comparison between control (a columns) and phentolamine-treated birds. ***$P<0.001$; **$P<0.01$; *$P<0.05$ (Student's *t* test).

O_2 consumption (readings at 4 min intervals) during 20 min preceding phentolamine or NA injection were calculated, and individual measurements throughout an experiment were plotted as the difference from this mean. Baseline values of O_2 consumption ranged between 2.01 and 3.10 ml min^{-1} 100 g^{-1}, and of *tb* between 37.9 and 41.1°C. *tf* varied between ca. 20° and 30°C.

Drugs used were: (−)-noradrenaline (L-arterenol bitartrate, Sigma) dissolved in 0.85% saline and phentolamine (Regitine, Ciba). The dose of NA (2 mg/kg) refers to base.

Results

Figure 1 summarizes the results obtained. Measurements were made on 8 birds but, due to dislodgement of the cannula in a few birds, not all the experiments could be accomplished according to the planned schedule.

When injected alone, NA (2 mg/kg i.m.) induced hypothermia and a fall in O_2 consumption, responses very similar to those observed earlier (Hissa *et al.*, 1975c). The maximum mean falls of *tb* and O_2 consumption were 2.6°C and ca. 9% (10 experiments in 7 pigeons, Figure 1a), respectively.

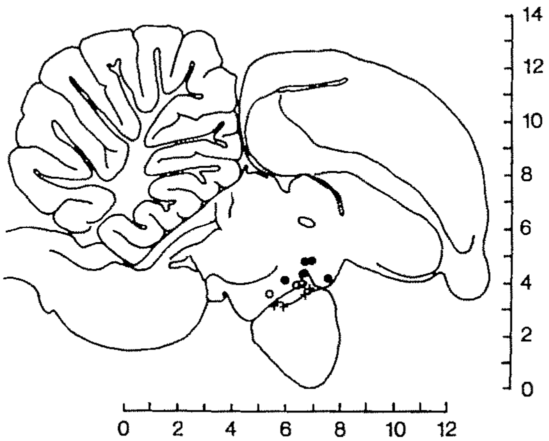

Figure 2 Injection sites of phentolamine in the pigeon brain shown at 1.0–2.0 mm lateral to the midline. (●) Prevention or attenuation of noradrenaline (NA, i.m.)-induced hypothermia; (○) no effect on hypothermia; (+) death after NA. The drawing is based on the Karten & Hodos (1967) atlas with the modification of mm scale to fit the smaller brain size of the birds used in the present work.

Following injection of phentolamine (20 µg/2 µl) into the hypothalamus and NA (2 mg/kg i.m.) 2 min later, irregular responses were seen. In 3 pigeons (7 experiments) a slight rise of *tb* and in 4 pigeons (9 experiments) a small fall was noted. In each case, a parallel change in O_2 consumption was seen, suggesting that the change in *tb* was related to metabolic heat production. The mean effect for the group was a fall of 0.1°C in *tb* and a rise of about 0.6% in O_2 consumption (Figure 1b).

The injection of NA (2 mg/kg i.m.) 10 min after intrahypothalamic injection of phentolamine (20 µg/2 µl) resulted in falls of *tb* and O_2 consumption in 11 experiments on 6 pigeons. A fall was noted in one other experiment on a pigeon which also responded with slight increases of *tb* and O_2 consumption in two experiments. When 10 µg/µl of phentolamine was injected into the hypothalamus and NA (2 mg/kg i.m.) injected 20 min later, hypothermia developed in 5 pigeons (10 experiments) and in one bird only (one experiment) a slight rise in *tb* and O_2 consumption was seen. The maximum mean falls over the 2–2.5 h experimental period for these two groups were 0.8°C and 1.3°C in *tb* and ca. 4% and 7% in O_2 consumption, respectively (Figure 1 c and d). The injection of saline (either 1 µl or 2 µl) into the hypothalamus did not affect the hypothermic responses elicited by intramuscular NA.

There were great individual variations among the pigeons with respect to shivering. After NA alone, shivering was absent for 15 min on average.

Generally, phentolamine pretreatment reduced this period and following 20 μg phentolamine 2 min before NA, shivering was maintained throughout, although slightly depressed in intensity.

Although the changes of *tf* were variable, intramuscular NA usually caused a fall of 4–8°C if the initial level was high (28–32°C) at the time of injection. When initially low (below and around 20°C), a transient rise (2–8°C) of *tf* was often seen.

Phentolamine itself seemed to have only a small effect on *tb*. The dose of 10 μg/μl caused a delayed slow rise (0.6 ± 0.07°C, $n=5$), and the maximum was reached at a latency of 60 to 80 min after injection. On the other hand, the dose of 20 μg/2 μl lowered *tb*, the fall averaging 0.6 ± 0.28°C ($n=5$) and the maximum being reached 30–95 min after administration. No noteworthy effect on shivering or O_2 consumption was seen.

Five pigeons (not included in the results presented above) died in the first experiment (20 μg/2 μl phentolamine injected 2 min before NA). The birds seemed to have difficulties in breathing and death occurred within 10–20 min after NA administration. At that time the body temperature exhibited no abnormal deviations from those usually observed in connection with NA injections. The injection sites in these birds are included in the mapping of cannula locations shown in Figure 2.

Discussion

Intrahypothalamically applied phentolamine caused an attenuation of the hypothermic effects of the intramuscular injection of NA. It has previously been shown that pretreatment with phentolamine prevents hypothermic effects of NA when both drugs are applied either into the hypothalamus (Hissa & Rautenberg, 1974) or intramuscularly (Hissa *et al.*, 1975c). Since different routes for phentolamine and NA were used in the present experiments, the basic question to be answered is whether the two drugs interact at the same site to cause the observed effects or, if different sites in both periphery and CNS are involved, how this could account for the results.

No specific data about the fate of intramuscular NA in pigeons can be given but, provided that NA binding in avian tissues is similar to that in mammals (Whitby *et al.*, 1961; Cohen & Rodriguez-Farre, 1975), a substantial proportion of the NA would be inactivated by binding to plasma proteins and tissue parenchyma. Among the peripheral effects of NA, a fall in *tf* presumably due to the constrictor effects on blood vessels in the legs was observed. Similarly, NA might affect shivering by reducing blood flow through the breast muscle. The possibility of a metabolic stimulatory effect of NA on the pigeon has been discussed previously (Hissa, Rantala & Jeronen, 1975b; Hissa *et al.*, 1975a, c; Pyörnilä *et al.*, 1976). Nevertheless, the primary effect of intramuscular NA is considered to be central and inhibitory on heat production at low *ta* (Hissa *et al.*, 1975a, c).

Injection of propranolol (i.m.) was shown not to affect the hypothermic effect of intramuscular NA at *ta* 6°C (Hissa *et al.*, 1975c). Assuming that propranolol enters the brain (see Masuoka & Hansson, 1967; Laverty & Taylor, 1968) and that the NA-induced hypothermia is mainly of central origin, the hypothermic response cannot then be attributed to *β*-adrenoceptor activation in the brain. In fact, mediation by *α*-adrenoceptors has been shown (Hissa *et al.*, 1975c).

Intrahypothalamic injection of 1 μg NA produced hypothermia in the pigeon and was blocked by a small amount of phentolamine (1–5 μg/μl) (Hissa & Rautenberg, 1975). Because of the small doses needed, it would appear that reversal of the hypothermic effect of intramuscular NA (2 mg/kg) by pretreatment with phentolamine (10 mg/kg i.m.) (Hissa *et al.*, 1975c) might also be due to a central effect, despite a rather poor passage of phentolamine into the brain (see Andén & Strömbom, 1974). On this basis, it was not surprising that the hypothermic effect of intramuscular NA was attenuated by phentolamine after direct blockade of *α*-adrenoceptors in the hypothalamus. On the whole, this evidence suggests that central stimulation of *α*-adrenoceptors is involved in the mediation of hypothermia induced by NA injected either centrally or peripherally into the pigeon at *ta* 6°C. Recently, however, Nisticò, Marley & Preziosi (1976) have demonstrated that in adult fowls antagonists at *β*-adrenoceptors may also prevent the hypothermic effects of catecholamines infused into the hypothalamus.

Intrahypothalamic phentolamine itself caused only a small and slow change in *tb*. On the assumption that NA is released continuously in the hypothalamus to control *tb* (Feldberg & Saxena, 1971), this might be explained by a rather low rate of release of NA at the time of injection (after ca. 2 h exposure of birds to *ta* 6°C). In view of the hypothermic effects of NA, this would not appear unreasonable. Chawla *et al.*, (1974) demonstrated a small and slowly developing hyperthermia following intraventricular injection of phenoxybenzamine in the pigeon at room temperature. Likewise, intraventricular phentolamine was shown to elevate *tb* in adult fowls at *ta* 6°C (Marley & Nisticò, 1975). This suggests that a greater release of NA from nerve terminals in the thermoregulatory pathway is needed in the maintenance of normal *tb* at *ta* around 20°C.

Variations in the location of the cannula might partly account for the variation of responses between different birds. However, no conclusions can be drawn without more accurate mapping. The locations of cannulae in pigeons that died were rather deep, bordering on optic chiasm and optic tract. The cause of death remains unestablished.

In conclusion, these experiments demonstrate that hypothalamic injection of phentolamine can cause a considerable attenuation of the hypothermic effects of an intramuscular injection of NA in the pigeon at *ta* 6°C. The results further suggest that the hypothermic effect of peripheral NA is mainly of central origin and due to an α-adrenoceptor agonist effect in the hypothalamus.

Please send reprint requests to A.P.

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(Received July 27, 1976.
Revised April 6, 1977)

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CARDIAC ADRENOCEPTORS AT LOW TEMPERATURE AND THE ADRENOCEPTOR INTERCONVERSION HYPOTHESIS

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1 The hypothesis that low temperature converts inotropic β-adrenoceptors to α-adrenoceptors has been tested in isolated heart preparations of the frog and rat.

2 The results do not support the adrenoceptor interconversion hypothesis. In the frog ventricle strip lowering the temperature from 24°C to 14°C did not significantly alter the inotropic potency of the sympathomimetic drugs isoprenaline, adrenaline and phenylephrine and did not reduce the potency of the β-adrenoceptor blocking drug propranolol as an isoprenaline antagonist. In the isolated left atrium of the rat lowering the temperature from 31°C and 24°C to 17–19°C did not significantly alter the inotropic potency of isoprenaline, noradrenaline and phenylephrine, did not diminish the potency of the β-adrenoceptor blocking drug propranolol and did not increase the potency of the α-adrenoceptor blocking drug phentolamine.

3 Phenylephrine acted on α- and β-adrenoceptors in rat left atrium at 31°C and 24°C, but only on β-adrenoceptors at 17–19°C.

Introduction

Adrenoceptors can be classified into two types, α or β, according to their sensitivity to agonists and antagonists. It has been suggested that α- and β-adrenoceptors are 'conformations' of a single adrenoceptor (Kunos & Szentivanyi, 1968; Kunos, Yong & Nickerson, 1973; Kunos & Nickerson, 1976). The following observation in the isolated heart of the frog has been accepted as proof of receptor interconversion (Kunos *et al.*, 1973; Kunos & Nickerson, 1976). The α-adrenoceptor blocking drug phenoxybenzamine inhibits the inotropic effect of adrenaline at low temperature and not at higher temperature; however, the adrenaline effect is inhibited at high temperature if the heart is pretreated with phenoxybenzamine at low temperature. It is assumed that at low temperature phenoxybenzamine irreversibly blocks the adrenoceptor in 'α-conformation' and that temperature elevation cannot convert it to the 'β-conformation'.

The problem is that one needs a very high concentration of an α-adrenoceptor blocking drug to inhibit the adrenaline effect on the frog heart at low temperature, which raises the question whether the inhibition is due to adrenoceptor blockade. High concentrations of α-adrenoceptor blocking drugs have inhibitory effects on the heart which are unrelated to adrenoceptor blockade.

It has further been reported that at low temperature the β-adrenoceptorblocking drug propranolol loses 90% of its potency as an antagonist of the inotropic effect of adrenaline on the frog heart (Kunos & Nickerson, 1976). The problem is that the time of treatment with propranolol was very short, which raises the question whether the receptor blockade was complete. Propranolol needs a considerable amount of time to exert its full effect at normal temperature, and low temperature further slows the development of the blockade.

Results similar to those found in frog heart have been reported from studies in isolated heart of rat (Benfey, Kunos & Nickerson, 1974).

This paper examines the evidence for the adrenoceptor interconversion hypothesis.

The adrenoceptor is a descriptive term and its nature is unknown. A response is said to be mediated by α-adrenoceptors when the relative potency of a series of agonists shows that adrenaline or noradrenaline is highest, phenylephrine is somewhat lower, and isoprenaline is very low, and when the response is susceptible to inhibition by a relatively low concentration of an α-adrenoceptor blocking drug. A response is considered to be mediated by β-adrenoceptors when measurement of the potency of a series of agonists shows that isoprenaline is highest and phenylephrine lowest, and when the response is susceptible to inhibition by a relatively low concentration of a β-adrenoceptor blocking drug.

Using these criteria I have found no evidence that low temperature converts inotropic β-adrenoceptors in frog heart to α-adrenoceptors (Benfey, 1975, 1976). It has also been reported that adenylate cyclase-coupled β-adrenoceptors in frog, rat and dog heart do not change with temperature (Benfey *et al.*, 1974; Caron &Lefkowitz, 1974).

Kunos *et al.* (1973), Benfey *et al.* (1974) and Kunos & Nickerson (1976) did not work under 'optimal conditions in experiments for the pharmacological characterization of adrenoceptors in isolated tissues' (Furchgott, 1972). Thus in tissues containing both receptors the potency of the β-adrenoceptor blocking drug should have been determined after blockade of α-adrenoceptors. The tissue uptake processes which affect the potency of adrenaline and noradrenaline should have been blocked. The rate of the frog isolated heart should have been kept constant by pacing when the inotropic effect was recorded, because sympathomimetic drugs can exert indirect inotropic effects through chronotropic actions.

Methods

Strips of frog ventricle were suspended in a solution of the following composition (mM): NaCl 111, $NaHCO_3$ 2.4, KCl 1.9, $CaCl_2$ 1.1, NaH_2PO_4 0.07 and disodium edetate 30 µM. Contractions were elicited with square-wave pulses (1–3 ms, voltage just above threshold) at a rate of 0.3 Hz (24°C) or 0.2 Hz (14°C) and recorded isometrically. The experiments were done between the months of September and February.

Strips of rat left atrium were suspended in a solution consisting of (mM): NaCl 114.9, $NaHCO_3$ 24.9, KCl 4.7, $CaCl_2$ 1.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 10 and disodium edetate 30 µM which was equilibrated with 5% CO_2 in O_2. Contractions were elicited with square-wave pulses (0.3–1 ms, voltage just above threshold) at a rate of 1 Hz (31°C), 0.5 Hz (24°C), or 0.3 Hz (17–19°C) and recorded isometrically. The rat atrium often did not respond to the sympathomimetic drugs at 17°C; the temperature was then raised to 18 and 19°C, and even then an inotropic effect was not always obtained.

Before exposure to drugs the heart preparations were maintained at a given temperature for at least an hour. Incubation with propranolol or phentolamine was for 1 h or more. The potency of the agonists was determined from cumulative dose-effect curves, a new agonist dose being added after the previous dose had reached its full effect. The potency of the agonists is expressed as the $-\log ED_{50}$ (M), which is the $-\log$ of the molar concentration that produces a half-maximal effect. The potency of the antagonists is expressed as the dose-ratio, which is the ratio of the potency of the agonist in the presence of the antagonist and in its absence.

he drugs used included: (−)-isoprenaline bitartrate dihydrate, (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate (Winthrop), (−)-phenylephrine hydrochloride (K & K Laboratories), (±)-propranolol hydrochloride (Inderal; Ayerst, McKenna & Harrison), and phentolamine methanesulphonate (Ciba).

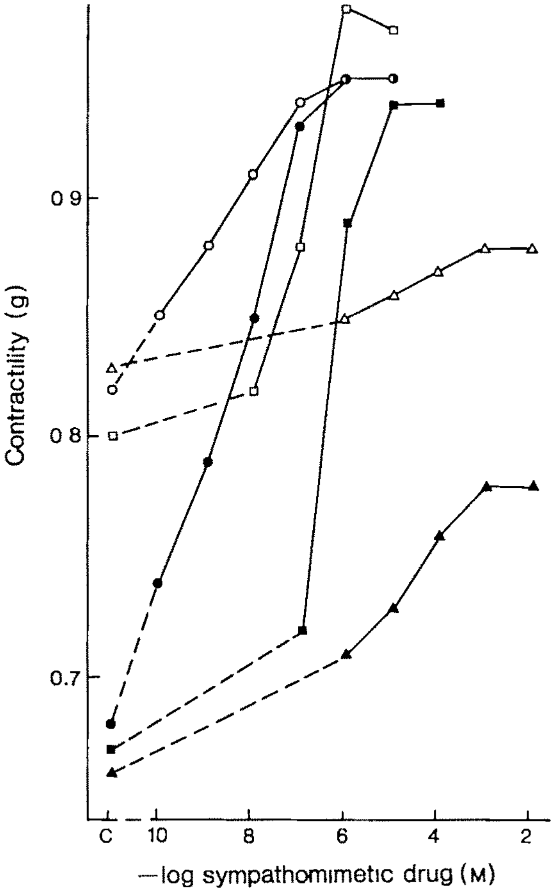

Figure 1 Effects of isoprenaline (○), isoprenaline in the presence of propranolol 0.34 µM (□), and phenylephrine (△) on contractility of frog ventricle strip at 14°C (open symbols) and 24°C (solid symbols). Means of 4–9 experiments. C: control contractility.

Results

Frog ventricle

Figure 1 shows that the contractility of the frog ventricle strips was higher at 14°C than at 24°C. Consequently, the inotropic effect of the sympathomimetic drugs was smaller at the low temperature than at the higher temperature. The

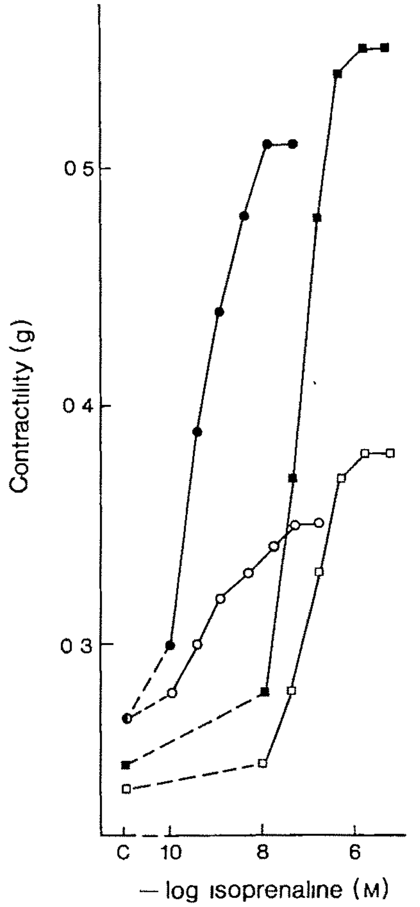

Figure 2 Effects of isoprenaline in the absence (○) and presence of propranolol 0.1 μM (□) on contractility of rat left atrium at 17–19°C (open symbols) and 31°C (solid symbols). Means of 5 experiments. C: control contractility.

inotropic effect of adrenaline was similar to that of isoprenaline, but that of phenylephrine was smaller, both at 24°C and at 14°C.

Lowering the temperature did not significantly change the potency of isoprenaline, adrenaline and phenylephrine. Isoprenaline remained the most potent, and phenylephrine the least potent drug. The potency of propranolol as an isoprenaline antagonist did not change with temperature.

Rat atrium

Lowering the temperature from 31°C to 17–19°C did not change the level of the contractility of the rat atrium but reduced the effect of the inotropic drugs (Figures 2, 3 and 4). The effect of phenylephrine was similar to that of isoprenaline and noradrenaline.

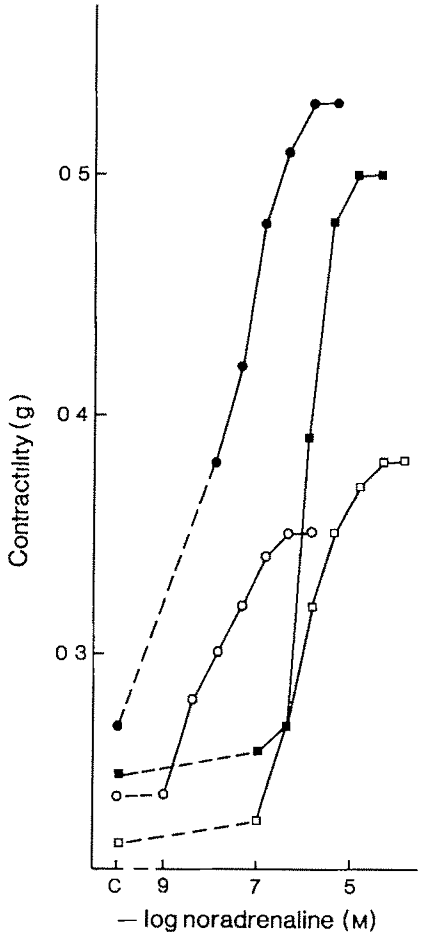

Figure 3 Effects of noradrenaline in the absence (○) and presence of propranolol 0.1 μM (□) on contractility of rat left atrium at 17–19°C (open symbols) and 31°C (solid symbols). Means of 5 experiments. C: control contractility.

The potency of isoprenaline, noradrenaline and phenylephrine and the potency of propranolol as an antagonist of isoprenaline and noradrenaline did not significantly change when the temperature was reduced from 31°C to 24°C and 17–19°C.

Phenylephrine behaved unexpectedly. The inotropic effect of phenylephrine was inhibited by propranolol and phentolamine at 31°C and 24°C, but only by propranolol at 17–19°C.

These results suggest that in the rat atrium at 17–19°C isoprenaline, noradrenaline and phenylephrine act solely on β-adrenoceptors and that at 31°C and 24°C isoprenaline and noradrenaline act solely on β-adrenoceptors and phenylephrine acts on α- and β-adrenoceptors.

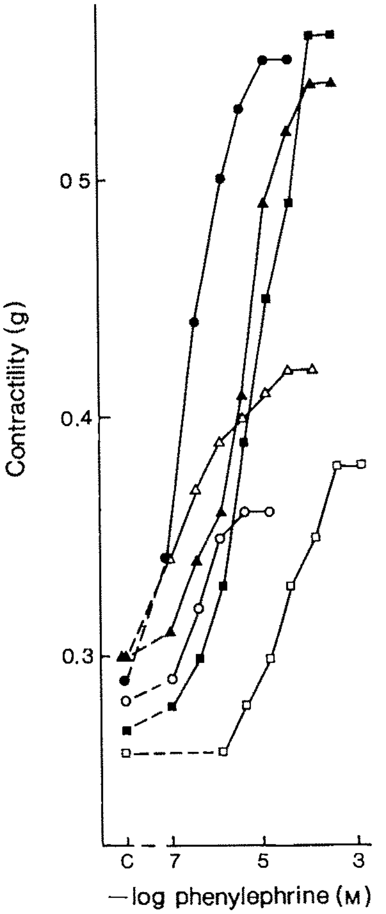

Figure 4 Effects of phenylephrine in the absence (O) and presence of propranolol 0.1 μM (□) and phentolamine 1 μM (Δ) on contractility of rat left atrium at 17–19°C (open symbols) and 31°C (solid symbols). Means of 5 experiments. C: control contractility.

Discussion

Amphibian hearts

There are four papers that deal with the potency of sympathomimetic drugs on the amphibian heart at low temperature, and their results are varied. For example, temperature reduction from 25°C to 12°C caused a tenfold increase in the chronotropic potency of adrenaline in the perfused toad heart (Harri, 1973) but temperature reduction from 24°C to 14°C led to a tenfold crease in the inotropic potency of adrenaline in the frog isolated heart (Kunos & Nickerson, 1976). At 12°C isoprenaline was more potent than phenylephrine in the perfused toad heart (Harri, 1973) and frog heart (Tirri, Harri & Laitinen, 1974); at 14°C isoprenaline was more potent than phenylephrine in the frog ventricle (Benfey, 1975).

There are three papers that deal with the potency of β-adrenoceptor blocking drugs on the amphibian heart at low temperature, and their results vary. For example, temperature reduction from 25°C to 12°C caused a fourfold decrease in the potency of propranolol as an isoprenaline antagonist in the perfused toad heart (Harri, 1973) and temperature reduction from 24°C to 14°C led to a slight increase in the potency of propranolol as an isoprenaline antagonist in the frog ventricle (Benfey, 1975). Temperature reduction from 25°C to 12°C caused a slight increase in the potency of propranolol as an adrenaline antagonist in the perfused toad heart (Harri, 1973) but temperature reduction from 24°C to 14°C led to a ten- to thirty-fold decrease in the potency of propranolol as an adrenaline antagonist in the isolated heart of the frog (Kunos & Nickerson, 1976).

Kunos & Nickerson (1976) treated their preparations with propranolol for only 10 min, and this does not appear to be a sufficient time to obtain a full receptor blockade, especially at low temperature. At 37°C it took 90 min to achieve a 98% equilibration with propranolol in guinea-pig isolated atria (Potter, 1967) and 75 min to obtain an 80–90% equilibration in guinea-pig isolated trachea (Furchgott, Jurkiewicz & Jurkiewicz, 1973). It is suggested that the reduction in potency of propranolol observed by Kunos & Nickerson (1976) is caused by an incomplete receptor blockade.

Turning to α-adrenoceptor blocking drugs: Nickerson & Nomaguchi (1950) reported that the chronotropic effect of adrenaline on the winter frog heart was inhibited by low concentrations of several β-haloalkylamines, e.g. dibenamine 3.4 μM; the inotropic effect was not inhibited. However, the blocking action of the β-haloalkylamines differed from their action on mammalian smooth muscle in that the blockade did not persist after the perfusion containing the active agent was replaced by fresh frog-Ringer solution.

Buckley & Jordan (1970) tested the adrenoceptor interconversion hypothesis by incubating the frog heart at 7°C with phenoxybenzamine 15 μM for 90 minutes. The chronotropic and inotropic effects of adrenaline were inhibited at 7°C, but the adrenaline antagonism disappeared when the temperature was raised to 24°C.

A persistent adrenaline antagonism was obtained by incubating the frog heart at 14°C with 7.3 μM phenoxybenzamine in four consecutive 10 min periods (Kunos *et al.*, 1973; Kunos & Nickerson, 1976). The effect was described as that of a 40 min incubation with 7.3 μM phenoxybenzamine, but it is that of a much higher concentration, or dose of the drug, because phenoxybenzamine cumulates and exerts progressively greater effects when administered repeatedly (Furchgott, 1966). Phenoxybenzamine is unstable in aqueous solution at neutral pH. Only part of the drug is available for reaction with tissues, and this part will alkylate a certain proportion of nucleophilic sites. Administration of fresh drug will continue this process.

The concentration of phentolamine used by Kunos & Nickerson (1976) to inhibit the inotropic effect of adrenaline (26.5 µM) also is much higher than that needed to block α-adrenoceptors in other tissues. The conclusion that the high concentrations of the α-adrenoceptor blocking drugs do not inhibit the adrenaline effect by adrenoceptor blockade is consistent with the observation that at low temperature phenoxybenzamine also inhibits the effect of isoprenaline (Benfey, 1975; Kunos & Nickerson, 1976), although there is evidence that isoprenaline does not act on α-adrenoceptors (Figure 1).

It is of interterest that phenoxybenzamine 2.9 µM inhibits the inotropic effect of acetylcholine on frog isolated ventricle (Benfey, 1975). Thus in the frog heart, phenoxybenzamine is a more potent antagonist of acetylcholine than of adrenaline.

Mammalian hearts

The mammalian heart posseses α-adrenoceptors, and α-adrenoceptors are present at normal temperature. Thus the inotropic effect of phenylephrine on the mammalian isolated heart is inhibited by α-adrenoceptor blocking drugs. α- And β-adrenoceptor mediated mechanisms differ; in contrast to β-adrenoceptor stimulation, α-adrenoceptor stimulation does not activate cardiac adenylate cyclase (Benfey, 1971; Osnes & Øye, 1975).

The concentrations of α-adrenoceptor blocking drugs which antagonize the inotropic effect of phenylephrine on mammalian heart are similar to those that block α-adrenoceptors in smooth muscle. The apparent dissociation constant of the receptor-inhibitor complex, K_B, for phentolamine (in the presence of propranolol 0.3 µM) was 28 nM in rabbit atrium (Benfey, 1973). This is in agreement with the pA_2 of 7.35 for phentolamine in rabbit papillary muscle (Schümann, Endoh & Wagner, 1974), and the K_B of 23 nM for phentolamine in rabbit aorta (Besse & Furchgott, 1976). Presynaptic α-adrenoceptors in guinea-pig atrium were blocked by phentolamine 0.31 µM (Langer, Adler-Graschinsky & Giorgi, 1977).

In the present study the K_B of phentolamine for phenylephrine antagonism was estimated (in the absence of propranolol) as 71 nM at 31°C and 49 nM at 24°C.

Higher concentrations of phentolamine can exert 'unspecific' effects. Thus phentolamine 3.1 µM inhibited the chronotropic effect of sympathetic nerve stimulation in guinea-pig atrium (Langer *et al.*, 1977).

Phenoxybenzamine is a highly potent α-adrenoceptor blocking drug. Phenoxybenzamine 1 nM inhibited the inotropic effect of phenylephrine on rabbit atrium (Benfey, 1973) and a 5 min incubation with phenoxybenzamine 0.3 nM inhibited the effect of noradrenaline on contraction of isolated spleen of the cat (Davidson & Innes, 1972). In the rabbit isolated aorta a 5 min exposure to phenoxybenzamine in a concentration of 29 nM or less significantly inhibited the effect of adrenaline (Nickerson & Chan, 1961). Dibenamine which is about 20 times less potent than phenoxybenzamine (Graham, 1962) had a pA_2 of 8.3 on 20 min exposure in guinea-pig vas deferens (Graham & Al Katib, 1966).

Higher concentrations of β-haloalkylamines have inhibitory effects on mammalian heart which are unrelated to adrenoceptor blockade. Thus 0.1 µM phenoxybenzamine inhibited the chronotropic and inotropic effects of acetylcholine on guinea-pig atrium (Benfey & Grillo, 1963); 0.78 µM phenoxybenzamine caused a 50% inhibition of neuronal noradrenaline uptake and 2.8 µM phenoxybenzamine caused a 50% inhibition of extraneuronal noradrenaline uptake in rat heart (Iversen, 1973); 10 µM dibenamine inhibited the chronotropic effect of isoprenaline (Krell & Patil, 1969), and 29 µM phenoxybenzamine inhibited the chronotropic effect of noradrenaline on guinea-pig atrium (Adler-Graschinsky, Langer & Rubio, 1972).

Benfey *et al.* (1974) reported that at 17°C four 10 min incubations with 7.4 µM phenoxybenzamine inhibited the inotropic effect of noradrenaline on rat atrium, but it is doubtful that the drug acted by adrenoceptor blockade in this high dose. Benfey *et al.* (1974) also found that lowering the temperature from 31°C to 17°C reduced the potency of propranolol as an antagonist of the ionotropic effect of noradrenaline on rat atrium, but the propranolol treatment was only done for 10 minutes.

Benfey *et al.* (1974) kept the driving rate at 1 Hz whereas I reduced it to 0.3 Hz at 17–19°C. Lowering the driving frequency should favour effects mediated by α-adrenoceptors. Thus lowering the driving frequency of the guinea-pig isolated ventricle from 2.5 to 1 Hz increased the inotropic effect of phenylephrine but not that of isoprenaline (Ledda, Marchetti & Mugelli, 1975); the inotropic effect of small concentrations of adrenaline on guinea-pig ventricle was inhibited by phentolamine at 1 Hz and by the β-adrenoceptor blocking drug practolol at 2.5 Hz (Mugelli, Ledda & Mantelli, 1976).

There are no other publications that deal with the potency of sympathomimetic or adrenoceptor blocking drugs on mammalian heart at temperatures below 25°C. It is of interest that lowering the temperature from 37.5°C to 17.5°C caused a more than ten-fold potentiation of the isoprenaline effect on guinea-pig isolated trachea (Foster, 1967).

Why did the high concentrations of the α-adrenoceptor blocking drugs inhibit the sympathomimetic drug effects at low temperature and not at higher temperature? It is probably easier to inhibit the small effect at low temperature than the greater effect at higher temperature. Low temperature greatly reduces the rate of formation of the active phenoxybenzamine metabolite, the aziridinium ion (Harvey & Nickerson, 1953; Rosen & Ehrenpreis, 1972). Effects which depend on formation of active metabolite are depressed by low temperature. Thus low temperature greatly reduced the acetylcholine antagonism in frog ventricle and guinea-pig atrium and the tyramine antagonism and noradrenaline potentiation in guinea-pig atrium (Benfey, 1975). When the rate of formation of the active metabolite is reduced, more of the highly lipid-soluble unchanged drug may accumulate in tissues and interfere with tissue activity.

In conclusion, the following objects are raised against the adrenoceptor interconversion hypothesis. First, the concentration of an α-adrenoceptor blocking drug needed to inhibit the inotropic effect of adrenaline on the frog heart at low temperature is very much higher than that which blocks α-adrenoceptors in mammalian heart and smooth muscle. Therefore, it is doubtful whether the inhibition of the adrenaline effect is due to adrenoceptor blockade. Second, the time of incubation with the β-adrenoceptor blocking drug was too short to ensure a full receptor blockade. Therefore, it is doubtful whether low temperature reduces the potency of a β-adrenoceptor blocking drug. Classical criteria of receptor identification have given no evidence that the frog heart possesses α-adrenoceptors.

This work was supported by a grant from the Medical Research Council of Canada.

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(Received September 6, 1976.
Revised March 28, 1977)

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MODIFICATION, BY ASPIRIN AND INDOMETHACIN, OF THE HAEMODYNAMIC AND PROSTAGLANDIN RELEASING EFFECTS OF *E. coli* ENDOTOXIN IN THE DOG

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1 Dogs treated with aspirin (10 mg/kg) or indomethacin (1.5 mg/kg) 45 min before, and 3 h after, an LD_{50} dose (1 mg/kg) of *E. coli* endotoxin were alive 72 h later.

2 Although all dogs in both treated groups survived, only those treated with indomethacin were protected against the fall in blood pressure 1–2 min following endotoxin.

3 Endotoxin increased the level of prostaglandin $F_{2\alpha}$ in both the mixed venous and arterial blood. No increase was observed in the aspirin and indomethacin-treated groups.

4 Aspirin and indomethacin treatment did not modify thrombocytopaenia or blood coagulation parameters following endotoxin.

Introduction

Non-steroidal anti-inflammatory drugs decrease the haemodynamic responses in endotoxin shock and enhance survival (Northover & Subramanian, 1962; Hinshaw, Soloman, Erdos, Reins & Gunter, 1967; Culp, Erdos, Hinshaw & Holmes, 1971; Greenway & Murthy, 1971; Hall, Hodge, Irvine, Katic & Middleton, 1972; Parratt & Sturgess, 1974; 1975a,b,c; 1976; Anderson, Jubiz, Tsagaris & Kuida, 1975a,b; Fletcher, Ramwell & Herman, 1976). These drugs have a variety of actions. They competitively inhibit 5-hydroxytryptamine release from platelets (Evans, Packham, Nishizawa, Mustard & Murphy, 1968; deGaetano, Donati & Vermylen, 1971; Hall *et al.*, 1972), stabilize lysosomes (Miller & Smith, 1966), inhibit the platelet release reaction (Zucker & Peterson, 1970; deGaetano *et al.*, 1971), decrease vascular permeability (Hinshaw *et al.*, 1967), and inhibit prostaglandin synthesis and/or release (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971). The exact mechanism by which these drugs exert a beneficial effect in endotoxin shock is unknown. The drugs may act by different mechanisms at a number of sites or by influencing a single mechanism which could mediate the pathophysiology of endotoxin shock.

The prostaglandins are a group of possible mediators which are known to cause haemodynamic derangements (Kloeze, 1966; Sorells, Erdos & Massion, 1971; Raflo, Wangensteen, Glenn & Lefer, 1973), alterations in platelet functions (Kloeze, 1966), changes in vascular permeability (Arora, Lahiri & Sanyal, 1969), and influence the inflammatory process (Crunkhorn & Willis, 1969). Blood levels of these potent vasoactive fatty acids are increased in endotoxin shock (Anderson, Jubiz, Fralios, Tsagaris & Kuida, 1972; Kessler, Hughes, Bennett & Nadela, 1973; Anderson *et al.*, 1975a,b; Fletcher *et al.*, 1976), and are related in time to the haemodynamic events (Fletcher *et al.*, 1976).

There have been two main problems in implicating the prostaglandins as mediators of the haemodynamic events during endotoxin shock in dogs. First, in most studies the prostaglandin levels were determined at arbitrary time intervals after giving endotoxin, rather than at the time observable haemodynamic changes were present. There are no studies described in which the prostaglandin levels were assessed immediately after endotoxin injection or at the time when the first detectable haemodynamic changes were present. Second, the relative degree of stress introduced in the model has often been inadequately defined. For example, the reported studies frequently did not state the lethality of the model. As a consequence, the relationship of the prostaglandins to the pathophysiology of endotoxin shock is not clear.

This study was designed to (1) determine the levels of prostaglandins present in the mixed venous and systemic arterial blood as soon as haemodynamic changes were present following endotoxin injection in an LD_{50} dog model, (2) evaluate the effects of pre- and post-treatment with aspirin or indomethacin on prostaglandin release, on the haemodynamic events, and on the survival, (3) investigate the possible relation of changes in prostaglandin concentrations with the coagulation, platelet, and complement abnormalities present in endotoxin shock.

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Methods

Adult male mongrel dogs (15–23 kg) were anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.), intubated and allowed to breathe spontaneously throughout the study. Indwelling catheters were inserted into the femoral and the pulmonary arteries through a cut-down in the right groin. The position of the pulmonary artery catheter (Swan-Ganz thermodilution) was monitored by pressure tracings and the femoral artery catheter was advanced into the descending aorta. The animals were kept in a supine position during the study. Cardiac outputs were measured by the thermal dilution method and calculated on a computer programme utilizing a PDP-12 computer (Digital Equipment Corporation). Systemic and pulmonary artery pressures were measured with transducers (Sanborn models 267AC and 267BC) and an 8-channel recorder (Sanborn model 958–100). All parameters were measured just before giving endotoxin and then 1–2, 15, 60, 120, and 240 min after the injection of endotoxin.

Blood analysis

Blood samples for prostaglandin analysis were collected from the femoral and pulmonary arteries simultaneously, and then immediately centrifuged at 4°C at 2450 rev/minute. The plasma was then removed and frozen (–20°C) until analysed. The thawed plasma (2 ml) was extracted with redistilled ethyl acetate (2 x 1 vol x 2) at pH 4.0. The dried ethyl acetate extract was reconstituted in benzene, ethyl acetate, methanol (60:40:10) and separated on a silicic acid column (0.5 gram). The prostaglandin F fractions were eluted with 5 ml benzene, ethyl acetate, methanol (60:40:30). Recovery of prostaglandin tracers added to plasma was 75–85% for prostaglandin $F_{2\alpha}$. Prostaglandin $F_{2\alpha}$ antisera was obtained from Dr H. Behrman (Yale, New Haven, Conn. U.S.A.). Radioimmunoassay was performed on the prostaglandin F fractions at two dilutions. Plasma standards were extracted with each group of unknown samples. The coefficient of variation for between and within assay reproducibility was required to be no greater than 10%. Data were calculated by the Rodbard and Hutt computer programme and expressed as picograms/ml plasma.

Arterial and mixed venous platelet counts were determined by an electronic particle counter (Coulter, Model ZBI). The one-stage method of Quick (1966) was used to assess the prothrombin time. The activated partial thromboplastin time was performed with Celite-activated phospholipid (Nye & Graham, 1962) and fibrinogen was measured turbidometrically (Parfentjev, Johnson & Clifton, 1953). Quantitation of the fibrin-split products was by means of the Staphylococcal clumping test (Hawiger, Niewearoski, Gusewich & Thomas, 1970). Total haemolytic complement (CH50) was determined as the greatest dilution of serum that produced 50% haemolysis in a standard sheep tanned erythrocyte microtitre plate system.

Intravenous injections

Endotoxin (*E. coli* 0 111:B4, Difco) was reconstituted in Ringer lactate solution on the day of the experiment. Aspirin (Mallinckrodt) was dissolved in sterile Ringer lactate solution with the aid of 0.1 N NaOH solution. Indomethacin (100 mg) was prepared in sterile 0.9% w/v NaCl solution (saline) by adding anhydrous sodium carbonate (37.5 mg) on the day of the experiment. Endotoxin, aspirin, and indomethacin were given intravenously.

Three groups of animals were studied: Group I (11 dogs) was given only an LD_{50} dose (1 mg/kg) of endotoxin; Group II (10 animals) was treated with aspirin (10 mg/kg), 45 min before, and 3 h after, endotoxin; Group III (10 animals) received intravenous indomethacin (1.5 mg/kg) 45 min before, and 3 h after, endotoxin.

No attempts were made to resuscitate the animals after endotoxin. All animals received a total of 400 ml of Ringer lactate solution during the study. Upon completion of the experiment, the dogs were placed in a recovery room, observed for 72 h and considered to be survivors if they were alive and well at this time.

Each dog was observed during a control period of 45 min before the administration of the endotoxin. At the end of the control period, baseline values were determined and endotoxin was injected into a peripheral vein. At the time of the acute fall in blood pressure, usually 1–2 min after endotoxin, measurements were taken and haemodynamic parameters assessed. In the aspirin (Group II) or indomethacin (Group III)-treated animals, there was an additional 45 min time period between giving the drugs and the administration of the endotoxin. Statistical analysis was performed by the paired two-tailed Student's *t* test for the difference between the baseline values and the experimental values in the same animals and the unpaired two-tailed *t* test for the difference between the groups. Chi square analysis was used to compare the survival data between the groups.

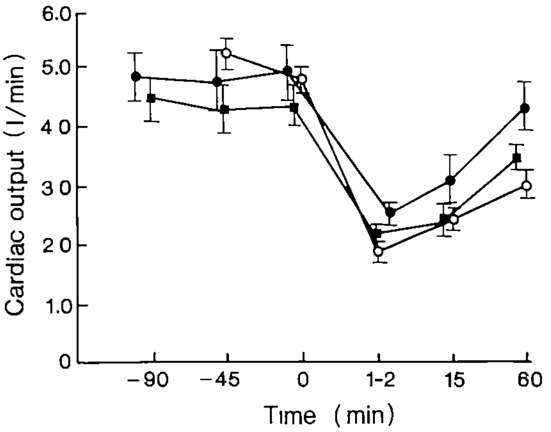

Figure 1 Mean cardiac outputs during endotoxin shock. Group I (○) consisted of 11 dogs given endotoxin alone. Group II (■) consisted of 10 dogs treated with aspirin (10 mg/kg) 45 min before, and 3 h after, an LD_{50} dose of endotoxin. Group III (●) consisted of 10 dogs treated with indomethacin (1 5 mg/kg) 45 min before, and 3 h after, an LD_{50} dose of endotoxin. The times indicate the following: −90 min (measurements taken immediately after surgical manipulation); −45 min (baseline measurements for all animals); 0 min (measurements in all groups immediately before endotoxin; in Groups II and III, the values indicate the direct effects of aspirin or indomethacin on cardiac output). The baseline cardiac output (5.0±0.3 l/min) is the mean±s.e. from all animals at −45 minutes. The other values represent mean values from the animals in each group Vertical lines show s.e. mean. Values at 120 min and 240 min were not different from values at 60 minutes.

Results

Survival

All (100%) animals that received aspirin (Group II) or indomethacin (Group III) survived, compared with only 46% in the endotoxin alone group. The survival rates were significantly ($P < 0.05$) different between the control group (I) and the treated groups (II and III).

Haemodynamic parameters

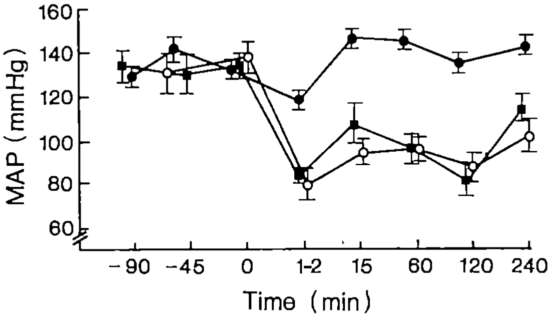

Figure 2 Mean systemic arterial blood pressures (MAP, mmHg) in dogs given only endotoxin (○) or given endotoxin and aspirin (■) or endotoxin and indomethacin (●). Endotoxin was given to each group at time 0 minutes. Vertical lines show s.e. mean. Baseline systemic arterial blood pressure was 130±7 mmHg (mean±s.e. mean).

Cardiac outputs for the three groups are shown in Figure 1. Neither aspirin nor indomethacin had any significant effect on cardiac output. All experimental values after endotoxin were significantly less ($P < 0.05$) than the baseline values except for the 60 min value in Group III. Cardiac output values after endotoxin for Group III were significantly ($P < 0.05$) higher than for Group I. The greatest decrease in cardiac output was observed in all groups 1–2 min following endotoxin. Interestingly, dogs in Group I that died (54%) had significantly ($P < 0.01$) decreased cardiac outputs (1.41±0.09 l/min) only at 1–2 min after the administration of endotoxin in comparison with those that survived (2.39±0.23 l/min). The animals that survived in this group (I) had cardiac outputs that were not different from the aspirin or indomethacin-treated dogs at any time.

Systemic mean arterial pressures (MAP) for all groups are shown in Figure 2. Neither aspirin nor indomethacin alone had any significant effect on MAP. Following endotoxin, mean arterial pressure values in the control (I) group of animals and the aspirin-treated (II) dogs were similar and were all significantly ($P < 0.01$) less than the baseline values. In contrast, after endotoxin the indomethacin-treated dogs (III) had MAP values that were not different from the baseline values and were significantly ($P < 0.01$) greater than the control (I) and aspirin-treated (II) groups. Group I dogs that died had significantly ($P < 0.01$) decreased pressures only at the 1–2 min interval (63±12 mmHg; mean±s.e.) when compared to those that survived (121±13 mmHg).

Pulmonary arterial pressures (PAP) are shown in Figure 3. Neither aspirin nor indomethacin alone had any significant effect on PAP. Only at the 1–2 min sampling time in the control (endotoxin alone) group did the PAP values increase significantly ($P < 0.05$) from the baseline values. Neither the aspirin (II) nor the indomethacin (III) group exhibited an acute rise in PAP as observed in the control group (I) at 1–2 minutes.

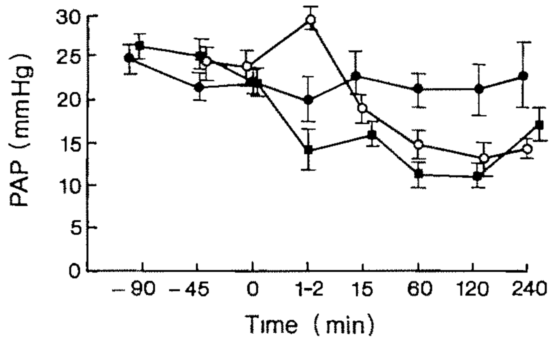

Figure 3 Mean pulmonary arterial pressures (PAP, mmHg) in dogs given only endotoxin (O) or given endotoxin and aspirin (■) or endotoxin and indomethacin (●). Endotoxin was given at time 0. Vertical lines show s.e. mean. Baseline pulmonary arterial pressure was 23 ± 2 mmHg (mean ± s.e. mean).

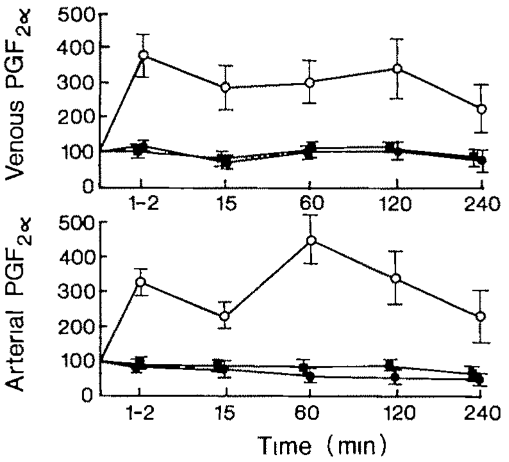

Figure 4 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) levels following the administration of endotoxin alone (O), of endotoxin following aspirin (■) and of endotoxin following indomethacin (●). Values are % of baseline values. Vertical lines show s.e. mean. Baseline value for venous prostaglandin $F_{2\alpha}$ was 242 ± 31 pg/ml; baseline value for arterial prostaglandin $F_{2\alpha}$ was 148 ± 20 pg/ml (means ± s.e. mean). For details of treatment see text. Treatment with either aspirin or indomethacin completely prevented the increase in circulating prostaglandin $F_{2\alpha}$ that resulted from endotoxin administration.

Platelets, coagulation and complement

The arterial and venous platelet counts were significantly ($P < 0.001$) decreased after endotoxin administration at all sampling times and in all groups; there were no significant differences between the mixed venous and arterial platelet counts in any group for the entire study. Coagulation tests demonstrated similar prolongations of the prothrombin time and activated partial thromboplastin time and there was a decrease in the fibrinogen concentration. Fibrin-split products were increased in all groups from the 60 min sampling time throughout the remainder of the study. There were no significant differences between the groups for these parameters at any sampling time.

Indomethacin alone (III) significantly ($P < 0.01$) decreased the total haemolytic complement levels when compared to the control (I) and the aspirin (II) treated groups. In addition, total haemolytic complement levels were significantly ($P < 0.01$) less at the 15 and 60 min sampling times in the indomethacin-treated (III) group when compared to the control (I) and the aspirin-treated (II) groups.

Prostaglandins

The systemic arterial and mixed venous prostaglandin $F_{2\alpha}$ values are shown in Figure 4. Before the administration of aspirin and indomethacin they were 126 ± 10 and 220 ± 34 pg/ml, respectively. Following

Table 1 Prostaglandin $F_{2\alpha}$ levels in control animals after endotoxin

Time (min)	*0*	*1–2*	*15*	*60*	*120*	*240*
Mixed venous						
Lived	209 ± 69 pg/ml	345 ± 94	233 ± 60	287 ± 136	341 ± 112	241 ± 109
Died	219 ± 60 pg/ml	382 ± 51	268 ± 80	266 ± 84	260 ± 118	218 ± 85
Arterial						
Lived	208 ± 20 pg/ml	315 ± 30	181 ± 35	362 ± 162	344 ± 180	238 ± 171
Died	209 ± 59 pg/ml	277 ± 82	201 ± 45	463 ± 200	242 ± 149	182 ± 50

Values are % of baseline values (mean with s.e. mean)
No significant differences noted between those animals that lived or died.

aspirin or indomethacin the arterial prostaglandin F_{2a} levels were 183 ± 30 pg/ml and 159 ± 20 pg/ml, and the mixed venous prostaglandin F_{2a} levels were 284 ± 30 and 213 ± 46 pg/ml, respectively.

The arterial and mixed venous prostaglandin F_{2a} levels were significantly ($P < 0.02$, at all sampling times) increased by endotoxin only in the control group (Figure 4), but there were no significant differences in either the arterial or the mixed venous prostaglandin F_{2a} concentrations between those animals that lived or died (see Table 1). The greatest increase in the mixed venous prostaglandin F_{2a} level was within 1–2 min after the injection of endotoxin whereas the peak of the arterial prostaglandin values was at 60 minutes.

Discussion

The important findings in this study are: (i) Therapeutic rather than pharmacological, doses of aspirin or indomethacin are equally effective in improving survival during endotoxin shock. (ii) That prostaglandin levels were elevated within 1–2 min after the injection of endotoxin and that this coincided with the first haemodynamic changes. (iii) Aspirin and indomethacin afforded significantly different degrees of attenuation of the haemodynamic events even though both were equally effective inhibitors of the increase in circulating prostaglandin F_{2a} evoked by endotoxin. (iv) That aspirin and indomethacin did not modify the thrombocytopaenia or the coagulation changes during endotoxin shock. (v) Animals that died in the control group did not have significantly higher levels of circulating prostaglandin F_{2a} than those animals that survived.

All previously reported studies (Northover & Subramanian, 1962; Hinshaw *et al.*, 1967; Parratt & Sturgess, 1974; 1975a,b,c) using aspirin or indomethacin-treatment employed varying doses (aspirin 20–200 mg/kg, indomethacin 10–20 mg/kg) given at various times (30 min before to 2 h after endotoxin administration). Pharmacological doses of these drugs have a variety of effects as described earlier. Since aspirin (10 mg/kg) and indomethacin (1.5 mg/kg) have been shown to be effective inhibitors of the synthesis of prostaglandins as early as 1 h after oral administration (Kocsis, Hernandovich, Silver, Smith & Ingerman, 1973), these lower doses were selected in the present study in the hope that they would be more specific inhibitors of the synthesis of prostaglandins (Flower, 1974). The times chosen for these doses (4 h apart) were based on the fact that the half-life of intravenously administered indomethacin in the dog is 3.6 h (Hucker, Zacchei, Cox, Broadie & Cantwell, 1966).

Treatment of endotoxin shock with anti-inflammatory drugs was first introduced by Northover & Subramanian (1962). They showed that the administration of anti-inflammatory drugs either before or after endotoxin resulted in improved circulatory function. Others (Hinshaw *et al.*, 1967; Greenway & Murthy, 1971; Culp *et al.*, 1971; Parratt & Sturgess, 1974; 1975a,b,c; 1976) have demonstrated improved haemodynamic function as well as increased survival by pretreatment with anti-inflammatory drugs. In contrast, Hinshaw *et al.* (1967) and Parratt & Sturgess (1975d) were unable to show increased survival when analgesic-antipyretic drugs were administered only after endotoxin. These studies appeared to indicate that pretreatment was necessary to improve survival. However, one must remember that these authors were investigating different species (cats and dogs) and that the degree of shock was severe (LD_{80}). On the basis of their studies it can not be concluded necessarily that treatment with aspirin or indomethacin in endotoxin shock will not improve survival. In our previous study (Fletcher *et al.*, 1976) we demonstrated that pre- and post-treatment with aspirin or indomethacin were not effective in improving survival in an LD_{100} endotoxin shock baboon model. We concluded that the model was too severe for determining the effects of drugs on survival. The present study was designed to evaluate the effects of pre- and post-treatment in a less severe (LD_{50}) shock model and was a logical step in determining the role of prostaglandins in the pathophysiology of endotoxin shock.

The idea that the arachidonic acid – prostaglandin system participates in the pathophysiology of endotoxin shock is well founded. Thus, several investigators (Anderson *et al.*, 1972; Collier, Herman & Vane, 1973; Kessler *et al.*, 1973) have shown increased levels of prostaglandins following the administration of endotoxin. However, in these studies, prostaglandin levels were determined at arbitrary time intervals which were not necessarily related to changing circulatory function. In addition, the relative degree of haemodynamic stress on the animal in these studies often was not stated. The association of the prostaglandins with events after endotoxin has been best demonstrated by Parratt & Sturgess (1975a) in cats and Anderson *et al.* (1975) in calves in which they showed that the rise in pulmonary artery pressure was closely related to the presence of prostaglandin F_{2a}. The calf, however, is exquisitely sensitive to endotoxin (Anderson *et al.*, 1975) and thus may not be a good model to determine pathophysiological mechanisms. Nevertheless, the above studies all support the concept that prostaglandins are elevated in endotoxin shock. Our first study (Fletcher *et al.*, 1976), and the present one, clearly demonstrate that plasma prostaglandin F levels are significantly increased at times when haemodynamic changes are occurring. The inhibition of prostaglandin synthesis by indomethacin in this study suggests that the prostaglandins may mediate the early haemodynamic events in endotoxin shock.

The most interesting new observation to emerge from the present work was that indomethacin-treatment clearly improved the haemodynamic status after endotoxin more than did aspirin-treatment. In a review of the data from our lethal study in baboons, aspirin did not improve the early haemodynamic changes as well as indomethacin, although both were effective as inhibitors of the increase in the concentrations of prostaglandins. Aspirin and indomethacin may alter vascular smooth muscle sensitivity to circulating vasopressor agents (Aiken & Vane, 1973; Zimmerman, Ryan, Gomer & Kraft, 1973; Jackson, Johnson, Ng, Pye & Hall, 1974; Kadowitz, Joiner & Hyman, 1975). In addition, indomethacin could eliminate the postulated prostaglandin-mediated negative feedback inhibition of sympathetic neural activity (Hedqvist, 1970) as suggested by Parratt & Sturgess (1974). The increased levels of catecholamines present in endotoxin shock (Vick, 1964) might effect a greater vasoconstriction in the presence of indomethacin or aspirin. In contrast to the previous studies, the present study did not show improved circulatory function with aspirin treatment. The explanation may be that the dose used was 4 to 10-fold less and it was given at different times. Nevertheless, aspirin was effective in preventing prostaglandin release.

In this study we could not demonstrate any effect of aspirin or indomethacin on the thrombocytopaenia or in the disorders of coagulation seen in endotoxin shock. It is possible that the stimulus to aggregation of platelets by endotoxin was much greater than any protective effect aspirin or indomethacin might have had at these doses. The reason why indomethacin alone decreased the total haemolytic complement levels in this study is unexplained.

The only haemodynamic observations that correlated ($r = +0.96$) with death in the control animals were those that occurred 1–2 min after endotoxin administration. Although the prostaglandins were elevated at this time, there were no differences between those animals that lived or died. It may be that the prostaglandins are not important for survival, but are elevated coincidentally with the early haemodynamic events. This study also suggests that the early haemodynamic events in the dog may not necessarily be related to survival since the aspirin treatment did not alter these events despite the fact that all the animals survived. In consequence, the primary factor in survival in this model may not be the inhibition of the prostaglandin synthesis, but may be related to other actions of aspirin and indomethacin.

This study was supported in part by the Naval Medical Research and Development Command, National Naval Medical Center, Department of the Navy, Research Task No. MPN06.01.0041 and ONR contract No. N00014–69–A–0220–008. The opinions or assertions contained herein are private ones of the authors and are not to be construed as official or reflecting the view of the Navy Department or the naval service at large. The technical assistance of A.G. Disimone, G. Wulff, J. Butler, R. Coulter, M. Gideon, E. Harris, R. Brown, D. Villalas, and Charlsie Armstrong is greatly appreciated. For aid in preparing this manuscript, we wish to thank Nancy Hage.

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(Received November 23, 1976.
Revised March 25, 1977.)

Br. J. Pharmac. (1977), **61**, 183–188

MECHANISM OF NICOTINE-INDUCED RELEASE OF NORADRENALINE FROM ADRENERGIC NERVE ENDINGS

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1 A study of the mechanism of release of [^{3}H]-noradrenaline ([^{3}H]-NA) by nicotine from isolated vas deferens of the rat was made using incubation media of different ionic composition.

2 Nicotine (20 μg/ml)-induced release of [^{3}H]-NA was significantly potentiated in K^+-free Krebs solution as compared to that in normal Krebs-Ringer solution.

3 Nicotine-induced release of [^{3}H]-NA was significantly reduced in Na^+-deficient Krebs solution (containing only 11 mM Na^+) and was abolished in Na^+-free Krebs solution.

4 In totally depolarized tissues, nicotine failed to cause an outflow of [^{3}H]-NA but Ca^{2+} (5 mM) did so.

5 Nicotine required the presence of Ca^{2+} in the incubation medium to cause release of [^{3}H]-NA from adrenergic nerve terminals, the magnitude of release being dependent upon the concentration of Ca^{2+}.

6 Nicotine-induced release of [^{3}H]-NA was demonstrated in high Ca^{2+}, Na^+-free Krebs solution in which all Na^+ had been replaced with Ca^{2+}.

7 It is concluded that nicotine increases the membrane permeability to both Na^+ and Ca^{2+}. It is also suggested that the increase in permeability to Ca^{2+} alone is not sufficient but a local depolarizing action of nicotine is necessary to cause release of noradrenaline from adrenergic nerve endings.

Introduction

Nicotinic agents can generate and propagate impulses at the adrenergic nerve terminals (Ferry, 1963; Cabrera & Torrance, 1964), but it has been contended that nicotine-induced release of noradrenaline (NA) is not caused by these impulses (Jayasundar & Vohra, 1977). Bevan & Su (1972) using an isotope and a frozen section technique demonstrated that nicotine is concentrated, probably via a NA uptake process, in the nerve-containing layer of rabbit aortic strips. However, Westfall & Brasted (1972) reported that nicotine did not have to be taken up into the adrenergic neurone before being able to release NA. The exact mode of action of nicotinic agents on the adrenergic nerve terminals remains unknown. The present work was undertaken to investigate whether the release of NA from adrenergic nerve terminals by nicotine requires the presence of Na^+, K^+ and Ca^{2+} in the incubation medium and to determine whether nicotine causes local depolarization and/or increase in permeability to Ca^{2+} at adrenergic nerve terminals.

Methods

Male Wistar rats (200–300 g) were killed by cervical dislocation and the vasa deferentia were dissected out.

Release of [^{3}H]-noradrenaline ([^{3}H]-NA) by nicotine

Both vasa deferentia were slit open longitudinally and were placed together in a culture tube containing 3 ml Krebs-Ringer bicarbonate solution. The incubation medium was continuously gassed with a mixture of 95% O_2 and 5% CO_2 and its temperature was maintained at 36 ± 1°C. After equilibration for 1 h, the tissues were incubated for 15 min in a tube containing 10 μCi of [^{3}H]-NA ((±)-[7-^{3}H]-noradrenaline, New England Nuclear Corporation, Boston, Specific activity 13.6 Ci/mmol) in 3 ml normal Krebs solution. The incubation was terminated after 15 min by washing the tissues rapidly in a tube containing 3 ml of fresh Krebs solution and repeating the procedure. The tissues were washed at 10 min intervals for 90 min to ensure the elimination of extraneuronally bound [^{3}H]-NA. The tissues were then incubated in fresh Krebs solution every 5 min and these solutions were retained for determination of [^{3}H]-NA. When the tissues were to be exposed to nicotine (20 μg/ml) for 5 min, the alkaloid contained in 0.1 ml of Krebs solution was added to the tube.

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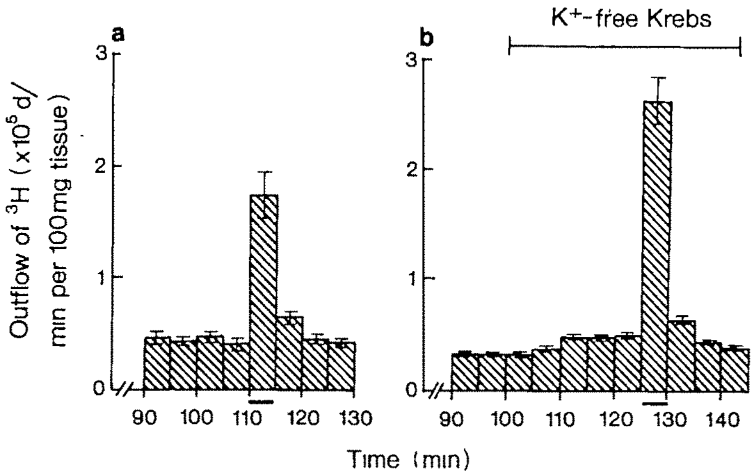

Figure 1 The effect of nicotine on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of the rat incubated in normal and K$^+$-free Krebs solution. The tissues were incubated with [^{3}H]-NA for a period of 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. (a) The tissues were exposed for 5 min to nicotine (20 µg/ml, indicated by a bar); (b) before exposure to nicotine, the tissues were incubated in K$^+$-free Krebs solution for 25 minutes The results are mean of 6 experiments. Vertical lines show s.e. mean.

Aliquots (0.5 ml) of each of the retained solutions were used to determine the total radioactivity. Counts per min (ct/min) were determined by the use of a Unilux II scintillation spectrometer and Bray's solution (Bray, 1960). The degree of quenching was determined by the dual channel ratio counting procedure.

Composition of incubation media

Normal Krebs-Ringer solution had the following composition (mM): NaCl 138.56, KCl 4.63, $CaCl_2$ 2.47, $MgCl_2$ 1.16, dextrose 11, disodium edetate 0.01 and ascorbic acid 0.005. This and the following modified Krebs solutions were buffered with Tris base (1 mM) and the pH was adjusted to 7.4–7.6. The composition of the following solutions remained the same as in normal Krebs-Ringer solution except for the modifications indicated below.

Na$^+$-free Krebs solution: all NaCl was replaced with an osmotically equivalent amount of sucrose (246.2 mM).

Na$^+$-deficient Krebs solution: all but 11 mM of NaCl was replaced with an osmotically equivalent amount of sucrose (226.7 mM).

K$^+$-free Krebs solution: KCl (4.63 mM) was omitted.

High K$^+$ Krebs solution: all but 45 mM of NaCl was replaced with 115 mM of KCl and $CaCl_2$ was reduced to 0.25 mM. This solution has a $Ca^{2+}/[Na^+]^2$ ratio which is the same as in normal Krebs solution.

Ca^{2+}-free Krebs solution: $CaCl_2$ (2.47 mM) was omitted.

Low and high Ca^{2+}-Krebs solution: $CaCl_2$ was either reduced to 0.25 mM or increased to 5 to 10 mM respectively. Since this involved an alteration of only small amounts of salts (up to 10 mM), a corresponding adjustment in tonicity of the solution was not made.

High Ca^{2+}, Na$^+$-free Krebs solution: all NaCl was replaced with 110 mM of $CaCl_2$.

Statistical analysis

Statistical differences were analysed by Student's *t* test. A value of $P < 0.05$ was considered significant.

Results

Effect of nicotine on the outflow of [^{3}H]-noradrenaline from tissues incubated in normal and K$^+$-free Krebs solution

As shown in Figure 1a, nicotine (20 µg/ml) increased the resting outflow of [^{3}H]-NA from tissues incubated in normal Krebs solution. The nicotine-induced outflow of [^{3}H]-NA was 420 ± 52% of the control resting outflow.

Figure 1b shows the effect of nicotine (20 µg/ml) in K$^+$-free Krebs solution. The total withdrawal of K$^+$ caused a gradual increase in the resting outflow of [^{3}H]-NA which was well sustained for at least 15 minutes. An exposure to nicotine at this time resulted in a marked increase in the outflow, which was 555 ± 44% of the control resting outflow of [^{3}H]-

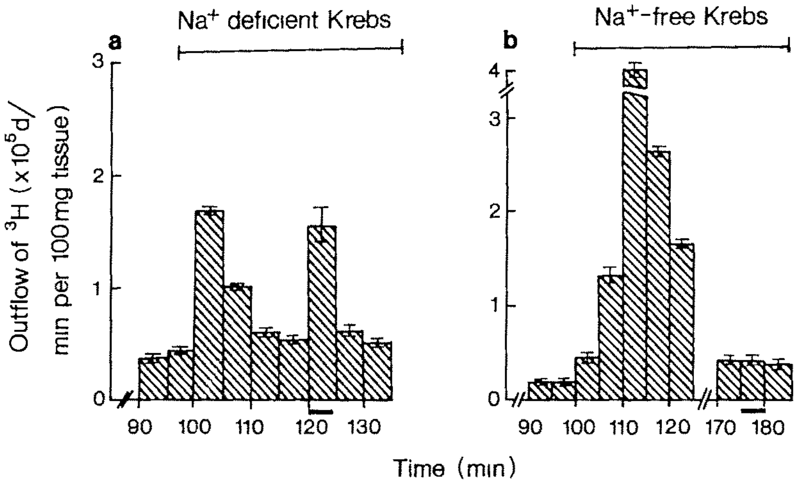

Figure 2 The effect of nicotine on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from the isolated vas deferens of the rat incubated in Na$^+$ deficient and Na$^+$-free Krebs solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. The tissues were incubated (a) in Na$^+$ deficient Krebs solution and (b) in Na$^+$-free Krebs solution. The tissues were exposed for 5 min to nicotine (20 μg/ml, indicated by a bar). The results are mean of at least 5 experiments. Vertical lines show s.e. mean.

NA. This is significantly ($P<0.05$) higher than the response obtained in normal Krebs solution.

Effect of nicotine in Na$^+$-deficient and Na$^+$-free Krebs solution

When tissues were exposed to a medium containing Na$^+$ 11 mM, the resting outflow of [^{3}H]-NA was enhanced but declined within 15 min almost to the initial level. Exposure to nicotine at this time resulted in an enhanced (Figure 2a) outflow which was 294±27% of the control resting outflow of [^{3}H]-NA. This response is significantly ($P<0.05$) lower than that obtained in normal Krebs solution.

The total withdrawal of Na$^+$ from the incubation medium caused a progressive increase in the resting

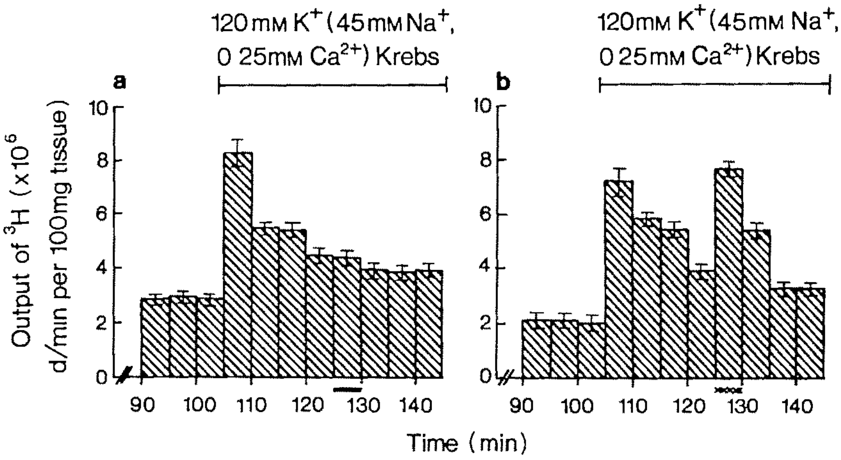

Figure 3 The effect of nicotine and calcium on the outflow of [^{3}H]-noradrenaline ([^{3}H])-NA) from isolated vas deferens of the rat incubated in high K$^+$-Krebs (120 mM K$^+$, 45 mM Na$^+$, 0.25 mM Ca^{2+}) solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. (a) The tissues were exposed to nicotine (20 μg/ml, indicated by a bar); (b) the concentration of Ca^{2+} in the incubation medium was raised to 5 mM (indicated by xxxx). The results presented are mean of at least 5 experiments. Vertical lines show s.e. mean.

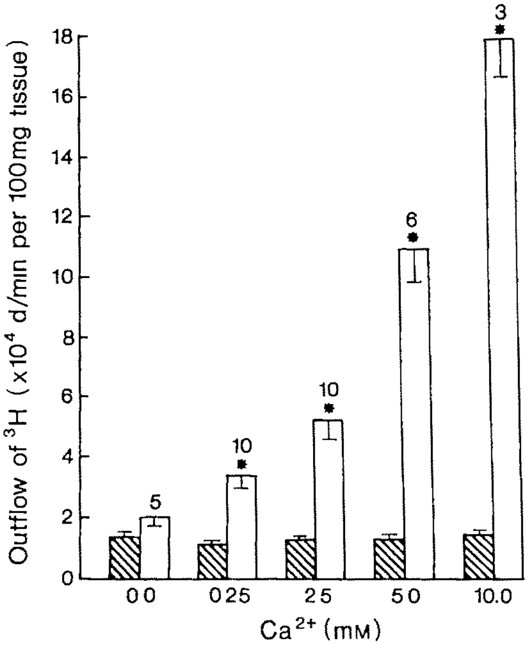

Figure 4 The effect of various concentrations of calcium on the nicotine-induced outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of the rat. The tissues were exposed to the Krebs solution containing different concentrations of Ca^{2+} for 20 min before the addition of nicotine (20 µg/ml). Control outflow—hatched columns; nicotine-induced outflow—open columns. The results presented are the mean of the number of experiments shown above the pairs of columns. Vertical lines show s.e. mean.
* Significantly different from the control resting outflow ($P<0.01$).

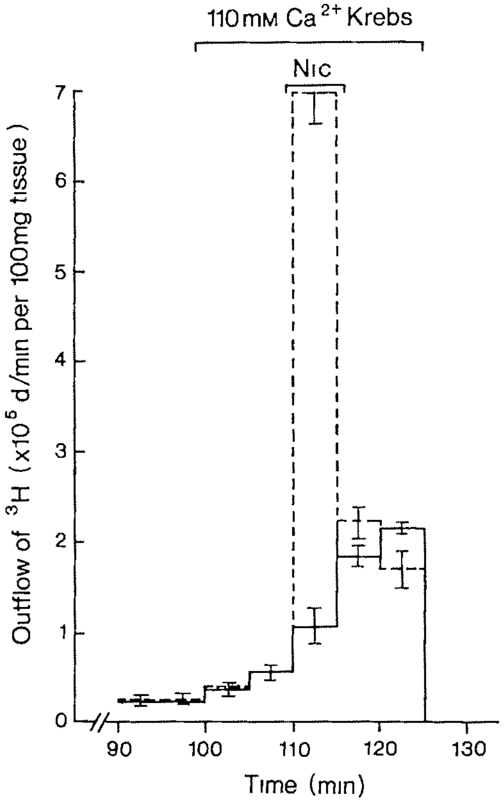

Figure 5 The effect of nicotine (Nic) on the outflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from isolated vas deferens of rat incubated in high Ca^{2+}, Na$^+$-free Krebs solution. The tissues were incubated with [^{3}H]-NA for 15 minutes. After the outflow of tritium attained a steady state (90 min), the tritium outflow per 5 min fraction was determined. The tissues were exposed to high Ca^{2+} (110 mM), Na$^+$-free Krebs solution indicated by a bar at the top of the figure. The solid line represents mean ± s.e. (6 control experiments). The broken line superimposed on the solid line represents mean ± s.e. (6 experiments) in which the tissues were exposed for 5 min to nicotine (20 µg/ml).

outflow of [^{3}H]-NA reaching a maximum in 15 minutes. The enhanced rate of release returned to the initial level in about 60 minutes. The addition of nicotine then failed to cause an increase in the resting outflow (Figure 2b).

Effect of nicotine in high K$^+$ Krebs solution

In preliminary experiments, it was seen that nicotine failed to cause an increase in the outflow of [^{3}H]-NA from tissues soaked in high K$^+$ Krebs solution. To test whether this failure to respond to nicotine in high K$^+$ medium was due to tissue damage, a response to calcium was chosen as the criterion, as it has been shown (Douglas & Rubin, 1963) that the addition of Ca^{2+} caused release of adrenaline from adrenal gland perfused in high K$^+$ medium. Calcium (up to 20 mM) failed to release [^{3}H]-NA from tissues immersed in high K$^+$ (containing 0 or 25 mM Na$^+$ and 2.5 mM Ca^{2+}) Krebs solution. When tissues were soaked in high K$^+$ Krebs solution in which the Ca^{2+}/[Na$^+$]^2 ratio was kept the same as in normal Krebs solution, the resting outflow of [^{3}H]-NA abruptly increased then declined but remained at a level higher than initially for at least 60 minutes. In this solution, addition of Ca^{2+} (5 mM) caused an increase in the outflow of [^{3}H]-NA (Figure 3b) which was 198 ± 8% of the control resting outflow. Nicotine (20 µg/ml) still failed to cause an increase in the outflow of [^{3}H]-NA (Figure 3a).

Effect of various concentrations of Ca^{2+} on nicotine-induced outflow of [^{3}H]-noradrenaline

The tissues were exposed to various concentrations of Ca^{2+} for 20 min before nicotine (20 µg/ml) was added.

The changes in the concentration of Ca^{2+} in the incubation medium did not alter significantly the rate of resting outflow of [^{3}H]-NA. The outflow of [^{3}H]-NA obtained before and in the presence of nicotine at various concentrations of Ca^{2+} is shown in Figure 4. In normal calcium medium (2.5 mM), the nicotine-induced release was 419 ± 45% of the control resting outflow. When Ca^{2+} concentration in the medium was reduced to 0.25 mM, there was a relatively smaller (299 ± 38% of control resting outflow) but significant ($P < 0.01$) increase in the outflow of [^{3}H]-NA caused by nicotine. In Ca^{2+} free medium, niotine caused only a small insignificant increase in the outflow of [^{3}H]-NA. When the concentration of Ca^{2+} was raised to 5 and 10 mM, the nicotine-induced outflow of [^{3}H]-NA was enhanced to 842 ± 97% and 1256 ± 89% of the control resting outflow respectively.

Effect of nicotine in high Ca^{2+}, Na^+-free Krebs solution

Addition of nicotine 15 min after exposure of tissues to high Ca^{2+}, Na^+-free Krebs solution caused a profound increase in the outflow of [^{3}H]-NA. As may be seen in Figure 5, at the time of exposure to nicotine, the resting outflow in control tissues was 1.07×10^5 d/min per 100 mg tissue wet wt, while the outflow in the presence of nicotine reached a peak of 6.9×10^5 d/min per 100 mg tissue wet weight. The difference between these values is highly significant ($P < 0.01$).

Discussion

It has been shown that nicotine-induced release of [^{3}H]-NA was potentiated in K^+-free Krebs solution as compared to that in normal Krebs solution. This suggests that the presence of K^+ in the extracellular fluid may not be necessary for the action of nicotine. The potentiated response to nicotine in K^+-free medium may be because of reduced reuptake of released noradrenaline (Gillis & Paton, 1967; Bogdanski & Brodie, 1969).

Nicotine-induced outflow of [^{3}H]-NA was significantly reduced in Na^+-deficient medium and was totally abolished in Na^+-free medium. These results suggest that nicotine requires at least a small amount of Na^+ for its action on the adrenergic nerve terminals. In Na^+-deficient and Na^+-free media, the inward movement of Na^+ (and hence depolarization of membrane) by any drug is expected to be impaired (Douglas & Rubin, 1963). Thus, the failing response to nicotine in Na^+-deficient and Na^+-free media observed in this study suggest that in normal Krebs solution, nicotine must increase the permeability of the nerve terminal membrane to Na^+ and consequently cause an influx of Na^+. This would result in local depolarization of the terminal membrane and cause the release of noradrenaline.

Nicotine failed to cause a release of [^{3}H]-NA in high K^+ Krebs solution, but the solution used in this study was considerably deficient in Na^+ (45 mM) and Ca^{2+} (0.25 mM). However, the failure of nicotine to release [^{3}H]-NA in this solution is unlikely to be due to the deficiency of Na^+ or Ca^{2+} because it was demonstrated that nicotine caused a significant increase in the outflow of [^{3}H]-NA in incubation media containing only 11 mM Na^+ (Figure 2a) or 0.25 mM Ca^{2+} (Figure 4). Incubation media containing high K^+ have been used to depolarize totally various isolated tissues such as cat adrenal gland (Douglas & Rubin, 1961; 1963), mammalian non-myelinated nerve fibres (Armett & Ritchie, 1963) and smooth muscles (Evans, Schild & Thesleff, 1958). If nicotine causes release of noradrenaline by depolarization of nerve terminal membrane, it would be expected to fail in totally depolarized tissues (i.e. in high K^+ Krebs solution). Since in the present study, nicotine did not cause release of [^{3}H]-NA in high K^+ Krebs solution, this further supports the contention that nicotine causes a local depolarization of nerve terminal membrane to bring about release of noradrenaline.

It has been demonstrated earlier that the release of noradrenaline following depolarization of adrenergic nerve endings was calcium-dependent and the magnitude of release was directly proportional to extracellular calcium concentration (Kirpekar & Wakade, 1968). Since depolarization of the cell membrane increases the influx of calcium ions in skeletal and cardiac muscles, adrenal medulla and squid axon (Hodgkin & Keynes, 1957; Bianchi & Shanes, 1959; Douglas & Poisner, 1962; Grossman & Furchgott, 1964), it was suggested that the depolarization of postganglionic adrenergic fibres may also lead to an increased influx of calcium (Kirpekar & Misu, 1967). In the present study, it was shown that the outflow of [^{3}H]-NA caused by nicotine is dependent upon the presence of Ca^{2+} in the medium and that the magnitude of the response is directly related to the extracellular concentration of Ca^{2+}. Thus nicotine may induce local depolarization which in turn causes influx of calcium, resulting in the release of noradrenaline.

The demonstration (Figure 5) that nicotine in high Ca^{2+}, Na^+-free Krebs solution causes a large outflow of [^{3}H]-NA, when local depolarization by nicotine is not possible suggests that nicotine is also capable of increasing permeability of membrane to Ca^{2+} to release [^{3}H]-NA. On the other hand, nicotine failed to cause a release of [^{3}H]-NA in Na^+-free and in high K^+ media, although these media contained enough Ca^{2+} to support nicotine-induced release of [^{3}H]-NA. Therefore, it seems that in a physiological ionic environment (normal Krebs solution), an increase in permeability to Ca^{2+} alone may not be sufficient to cause the release of noradrenaline. Thus a stronger stimulus by nicotine, i.e. the depolarization of nerve terminals seems to be necessary to bring about the release of noradrenaline.

It is concluded that nicotine increases the membrane permeability to both Na^+ and Ca^{2+}. It is also suggested that a mere increase in permeability to Ca^{2+} by nicotine is not sufficient but a local depolarizing effect of nicotine is necessary to cause release of noradrenaline from adrenergic nerve terminals. Nicotinic agents have earlier been shown to cause increase in permeability to Na^+ as well as Ca^{2+} at various nicotinic sites such as mammalian non-myelinited nerve fibres (Armett & Ritchie, 1963), neuromuscular junction (Del Castillo & Katz, 1955; Jenkinson & Nicholls, 1961) and adrenal medulla (Douglas & Rubin, 1961; 1963). Thus, the mode of action of nicotine on adrenergic nerve terminals resembles its action at other sites.

The authors wish to thank Dr Prasad D.M.V. Turlapaty for his reading and helpful criticism of the manuscript. The work was supported by funds from the Medical Research Council of Canada.

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*(Received December 15, 1976.
Revised May 3, 1977)*

Br. J. Pharmac. (1977), **61**, 189–197

LIGAND BINDING TO MUSCARINIC RECEPTORS IN INTACT LONGITUDINAL MUSCLE STRIPS FROM GUINEA-PIG INTESTINE

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1 The binding of ligands to muscarinic receptors in intact longitudinal muscle strips from guinea-pig small intestine has been determined by measuring the inhibition of the irreversible binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM).

2 The IC$_{50}$ values for inhibition of [^{3}H]-PrBCM binding by a given ligand were generally higher in intact strips than those reported for broken-cell preparations. This effect is probably due, at least in part, to the presence of an access-limitation factor in the kinetics of the irreversible binding of [^{3}H]-PrBCM to the intact tissue.

3 The mean Hill coefficients for antagonist binding approached unity, but those for strong agonists were significantly less than unity. There was, with the possible exceptions of hexyltrimethylammonium and oxotremorine, reasonably good agreement with the Hill coefficients reported for brain homogenates.

Introduction

Following the classic study of Paton & Rang (1965) on the binding of [^{3}H]-atropine by longitudinal muscle strips from guinea-pig intestine, a number of groups have demonstrated muscarinic receptor binding by tritium-labelled reversible or irreversible receptor ligands (see Birdsall & Hulme, 1976 for a review). Some of these studies, like the original, have used pharmacologically intact tissues, generally in conjunction with the use of an irreversible ligand (Rang, 1967; Cuthbert & Young, 1973; Fewtrell & Rang, 1973; Burgen, Hiley & Young, 1974a), but there are a number of experimental difficulties in this approach and most groups have preferred to work with tissue homogenates where equilibrium measurements with reversible ligands can be conveniently made and where statistical errors are generally small. The values of the binding affinities for antagonists obtained by the use of tissue homogenates are in general in excellent agreement with those determined from inhibition of the contractile response (Birdsall & Hulme, 1976). However, the point of particular interest that has emerged from ligand binding studies is that the binding of muscarinic agonists does not follow a single simple mass-action curve (Burgen & Hiley, 1974; Young, 1974; Birdsall, Burgen, Hiley & Hulme, 1976; Hulme, Burgen & Birdsall, 1976; Birdsall & Hulme, 1976). The binding of agonists is not readily correlated with the contractile response and in these circumstances measurements on a tissue which is demonstrably pharmacologically intact become important, in order to establish that the process of homogenization has not in some way modified agonist binding. So far as comparison has been made, largely limited to the binding of carbachol, the agreement has been good, but a more extensive examination of muscarinic ligand binding to an intact tissue, which we describe here, was clearly desirable.

Methods

[^{3}H]-Propylbenzilylcholine mustard

[^{3}H]-Propylbenzilylcholine mustard ([^{3}H]-PrBCM), specific activity 1.0 Ci/mmol, was prepared and allowed to cyclise to the aziridinium ion before use as described previously (Burgen *et al.*, 1974a).

Binding measurements

The procedure used here has been described in detail elsewhere (Burgen *et al.*, 1974a). Briefly, longitudinal muscle strips (6–13 per incubation, each usually weighing 3–8 mg) from guinea-pig small intestine were suspended in 400 or 800 ml Krebs–Henseleit solution at 30°C gassed with 5% CO$_2$ in O$_2$ and incubated for 1 h before addition of [^{3}H]-PrBCM (final concentration 2.2 nM). Unless specified otherwise, antagonists whose binding was to be studied were added 30 min, and agonists 1 min, before the [^{3}H]-PrBCM. Incubation with [^{3}H]-PrBCM (10 min) was terminated by transferring the strips briefly to 200 ml fresh Krebs and then to a further 200 ml Krebs at 30°C. Washing was continued for 75 min with two changes of the solution and the strips were then blotted dry, weighed, dissolved in Soluene (Packard) and the tritium determined by liquid scintillation counting.

Analysis of curves of inhibition of [^{3}H]-PrBCM binding

The curves of % of uninhibited binding of [^{3}H]-PrBCM *versus* concentration of inhibitor (e.g. Figure 1) can be considered to be made up of two components, an inhibitor-insensitive component presumed to represent non-receptor binding and a second component reflecting the receptor occupancy of the inhibitor. The time course of the receptor-specific binding of [^{3}H]-PrBCM approximates, over the first 40 min, to a single exponential function (Burgen *et al.*, 1974a), consistent with the reversible drug-receptor complex initially formed being converted into a covalently-bound complex in a step with a rate constant much greater than that for dissociation of the reversible complex (Gill & Rang, 1966). In the simplest case, assuming that the drug-receptor interaction is rate-determining, the total occupancy, p, which is equivalent in these circumstances to the irreversible binding measured, at any time t is given (Gill & Rang, 1966) by:

$$\ln(1-p) = -k_1 \cdot \mathrm{M} \cdot t$$

where k_1 is the rate constant for formation of the reversible complex and M is the concentration of the aziridinium ion derivative of [^{3}H]-PrBCM. In the presence of a reversible inhibitor with affinity K_a and at a concentration A, the occupancy, p_I, becomes

$$\ln(1-p_I) = \frac{-k_1 \cdot \mathrm{M} \cdot t}{1+A \cdot K_a}$$

Hence

$$\frac{\ln(1-p_I)}{\ln(1-p)} = \frac{1}{1+A \cdot K_a}$$

Expanding

$$\frac{-p_I-p_I^2/2 \cdots\cdots}{-p-p^2/2 \cdots\cdots} = \frac{1}{1+A \cdot K_a}$$

Hence when p is small, so that $p \gg p^2/2$, then

$$\frac{p_I}{p} = \frac{1}{1+A \cdot K_a}$$

(and the fractional inhibition, $1-p_I/p$, is equal to the occupancy of the antagonist). The proportion of the maximum specific binding achieved by [^{3}H]-PrBCM after 10 min incubation, equivalent on this analysis to p, is 0.16 and $p^2/2 = 0.013$, so that the condition that $p \gg p^2/2$ is apparently reasonably well satisfied.

The limitation of this simple approach is that it makes no allowance for diffusional delays. If there is an access-limitation factor then it is unrealistic to assume that the time-course of the irreversible binding of [^{3}H]-PrBCM can be approximated by a single exponential function with a rate constant of $1.4 \times 10^5 \mathrm{M}^{-1} \mathrm{s}^{-1}$ (Burgen *et al.*, 1974a). Reaction with [^{3}H]-PrBCM presumably occurs first with receptors on the outer surfaces of the superficial layer of cells and then subsequently as the drug diffuses inwards through the channels between cells, with receptors on cells in deeper layers — assuming that the distribution of receptors throughout the tissue is not grossly non-uniform. It seems likely that the rate constant for binding to the superficial receptors will be of the same order as that for homogenates, i.e. around $2.5 \times 10^6 \mathrm{M}^{-1} \mathrm{s}^{-1}$ (Burgen, Hiley & Young, 1974b) and that the same rate constant will describe the binding to the inner receptors, except that there the concentration of [^{3}H]-PrBCM will only slowly approach that in free solution. In this situation measuring [^{3}H]-PrBCM bound at a time short compared with that required to achieve saturation does not achieve a low occupancy of a homogeneous population but selects a group of receptors, largely on superficial cells, with which rapid reaction occurs. On the simple model the effect of a 10 min incubation period with a rate constant of $2.5 \times 10^6 \mathrm{M}^{-1} \mathrm{s}^{-1}$ instead of $1.4 \times 10^5 \mathrm{M}^{-1} \mathrm{s}^{-1}$ would be to shift the % of uninhibited uptake *versus* [inhibitor] curve along the concentration axis by a factor of about 4, with a small increase in the Hill coefficient, 1.01 → 1.12. In practice a somewhat smaller shift might be expected since for some receptors where the concentration of [^{3}H]-PrBCM is only just starting to rise the condition that $p \gg p^2/2$ will be much better satisfied. However, this analysis does suggest that even though using the simple approach may give poor estimates of binding affinities, it still provides a useful model against which the experimental curves can be tested to see whether they are consistent with a single mass-action equilibrium between inhibitor and receptor. To do this, inhibitor binding was assumed to follow a Hill equation, $A^n \cdot K_a/(A^n \cdot K_a + 1)$, and the best-fit values of n, the Hill coefficient, determined. The actual equation fitted, using the Patternsearch procedure of Hooke & Jeeves (1961; Colquhoun, 1971), was

$$\%\ \text{of uninhibited binding} = \frac{100-NS}{A^n \cdot K_a + 1} + NS$$

with n, K_a and NS (non-specific, i.e. non-receptor, binding) as unknown. Each point was weighted according to the reciprocal of the approximate variance associated with it. The Patternsearch method tended to work well where $n \sim 1$ but located subminima much more frequently when n was small. The best fit values were taken to be those that gave the lowest residual sum of squares after repeated trials with different initial parameter estimates. An alternative non-linear fitting procedure using a modified Marquardt approach as implemented in the Harwell Library routine VBOIA on the Cambridge IBM 370/165 gave similar values, where it succeeded, but failed to find a solution in some cases. The difficulties with both techniques probably arise from the fact that if $n \neq 1$ then almost certainly the binding curve of the inhibitor will not have an invariant value of n over its whole length and the numerical value of n obtained serves only as a useful index of the deviation of the binding curve from a single mass-action equilibrium. The use of the Hill equation has advantages, since the slope of the curve is the parameter of particular interest (Reiche & Zinke, 1974), but at the same time the limitations of the equation as a model to fit the data at low n may introduce problems of convergence and evaluation of error estimates (Reich, Winkler & Zinke, 1974). Consequently the mean Hill coefficients were also determined by linear regression analysis of conventional unweighted Hill plots of log $\{(100-\%$ of uninhibited binding$)/(\%$ of uninhibited binding$-NS)\}$ *versus* log $[A]$, ignoring points within 5% of the 100% or non-specific (taken as 13%) limits.

For analysis of agonist inhibition curves as binding to two independent sites, the equation fitted, using the Harwell VBOIA routine, was:

$$\%\text{ of uninhibited binding} = 100 - \frac{N1 . A}{A + K_1} - \frac{N2 . A}{A + K_2}$$

where A is the concentration of the inhibitor, K_1 and K_2 its dissociation constants for the two sites, and $N1$ and $N2$ the percentage of the total binding of [^{3}H]-PrBCM, after a 10 min incubation with the mustard, associated with each site. [^{3}H]-PrBCM is presumed not to distinguish between the two sites.

Organ bath experiments

Longitudinal muscle strips were suspended in 10 ml of Krebs–Henseleit solution, gassed with 95% O_2 and 5% CO_2, at 30°C in a conventional organ bath. Contractions were recorded isotonically. Agonists were in contact with the tissue for 15–30 s and doses were added at 3 min intervals.

Results

Binding of muscarinic antagonists

The effect of methylatropinium on the amount of [^{3}H]-PrBCM bound during a 10 min incubation period with longitudinal muscle strips is shown in Figure 1. The Hill coefficient for the apparent binding curve for methylatropinium, 0.99 (Table 2), is consistent with a simple mass-action equilibrium, but the apparent binding affinity, $2.4 \times 10^8\,M^{-1}$, is lower than that determined from inhibition of the contractile response, $2.1 \times 10^9 M^{-1}$ (Paton & Rang, 1965). A similar discrepancy, mirrored in the difference between the IC_{50} values in the intact strips and those reported for homogenates (Birdsall & Hulme, 1976; Yamamura & Snyder, 1974) exists for all the antagonists tested (Table 1) and is in the direction predicted if there is a diffusional factor in the kinetics of binding of [^{3}H]-PrBCM (cf Methods section). The extent of the shift for atropine is very similar to that apparent in the inhibition of [^{3}H]-BCM binding to muscle strips (Fewtrell & Rang, 1973). Whether the shifts can be wholly explained by diffusional effects is uncertain, but at least in the case of benzhexol, where the difference is particularly large, it seems likely that other factors are involved. If additional factors do exist then it seems equally likely that they involve some property of the intact muscle, since the binding affinities of antagonists to homogenates of mammalian cerebral cortex, measured using the same preparation of [^{3}H]-PrBCM (Burgen *et al.*, 1974b), agreed well with the values from organ bath studies.

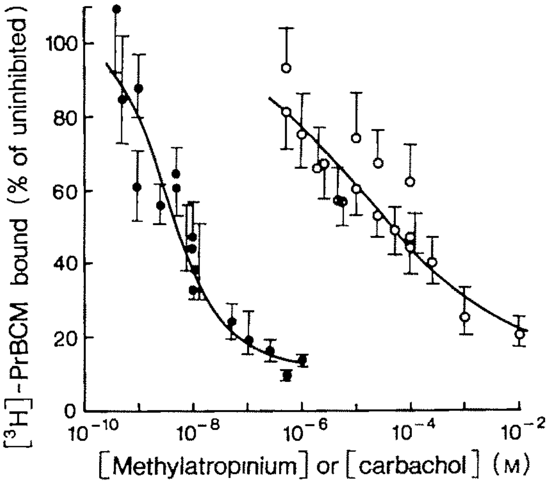

Figure 1 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips by methylatropinium and by carbachol. The experimental conditions and the method used to determine the weighted best-fit curves are described under Methods The data in this Figure were derived from measurements on some 600 muscle strips. Error bars represent the 69% confidence limits. (●) Methylatropinium; (O) carbachol.

The conclusion drawn from simple consideration of the effect of a diffusional barrier that the inhibition

Table 1 IC_{50} values for inhibition of receptor-specific [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by muscarinic ligands: comparison with broken cell preparations

		$IC_{50}(M)$		
			Homogenates	
	Non-specific binding (%)§	*Intact muscle*	*Cerebral cortex*†	*Longit. muscle*‡
Atropine§§	–	6×10^{-9}††	5.9×10^{-10}*	$3–4 \times 10^{-9}$
Benzhexol	11	1.2×10^{-7}††	7.1×10^{-9}	–
Methylatropinium	12	4.2×10^{-9}	3.5×10^{-10}*	$1–2 \times 10^{-10}$
Propylbenzilylcholine	9	4.0×10^{-8}	1.0×10^{-8}	–
Acetylcholine	11	8.9×10^{-6}	3.3×10^{-6}	–
Carbachol	15	2.0×10^{-5}	1.5×10^{-5}	$2–3 \times 10^{-6}$
(+)-Methacholine	(14)¶	1.3×10^{-5}	2.0×10^{-6}	$2–3 \times 10^{-6}$
Methylfurmethide	16	1.5×10^{-5}	2.2×10^{-6}	–
Oxotremorine	13	6.6×10^{-7}	4.6×10^{-7}	$5–8 \times 10^{-7}$
Pilocarpine	21	3.6×10^{-6}	6.0×10^{-6}	$7–9 \times 10^{-7}$
C_6TMA	14	1.7×10^{-5}	6.3×10^{-6}	–
	12	2.5×10^{-6}**		
C_{12}TMA	18	5.8×10^{-6}	–	–
	16	2.9×10^{-6}**		

Antagonists were preincubated with the muscle for 30 min, and agonists for 1 min, before [^{3}H]-PrBCM addition.

§ From weighted non-linear fit (see Methods); † Data taken from Birdsall & Hulme (1976); ‡ Data taken from Yamamura & Snyder (1974); §§ IC_{50} value for intact muscle taken from Burgen *et al* (1974a). †† For comparison with the values for [^{3}H]-PrBCM, IC_{50} values for inhibition of [^{3}H]-BCM binding to intact strips, estimated from the data of Fewtrell & Rang (1973) were: atropine, 5×10^{-9} M; benzhexol, 3×10^{-8} M * Value for (–)-isomer. ¶ Lower limit of the curve ill-defined (cf. text). Best-fit curve (Figure 3) obtained by setting non-specific binding of [^{3}H]-PrBCM to 14%. ** 30 min preincubation.

Abbreviations: C_6TMA, hexyltrimethylammonium; C_{12}TMA, dodecyltrimethylammonium.

Table 2 Mean Hill coefficients for muscarinic ligand binding: comparison with values from homogenates

	Mean Hill coefficients		
	Intact strips		
	*Non-linear fit**	*Hill plot**	*Homogenates with ref.***
Atropine	–	0.84 ± 0.06 (7)‡	~1.0 a
Benzhexol	1.00 (8)	0.88 ± 0.14 (6)	~1.0 a
Methylatropinium	0.99 (16)	0.96 ± 0.09 (13)	~1.0 a
Propylbenzilylcholine	0.90 (5)	0.93 ± 0.13 (4)	1.0 b
Acetylcholine	0.62 (10)	0.54 ± 0.06 (9)§	0.42, 0.50, 0.63, a,b,c
Carbachol	0.36 (18)	0.31 ± 0.05 (16)	0.33 a
(+)-Methacholine	0.46 (11)†	0.36 ± 0.06 (9)	0.52†† a
Methylfurmethide	0.58 (10)	0.53 ± 0.06 (10)	0.48 a
Oxotremorine	0.65 (17)¶	0.44 ± 0.06 (14)	0.88, 1.0 a,b
Pilocarpine	0.82 (8)	0.63 ± 0.12 (8)	0.95, 1.0 a,b
C_6TMA	0.55 (14)	0.53 ± 0.08 (13)	1.0 a
	0.72 (9)‡‡	0.82 ± 0.10 (8)	
C_{12}TMA	0.80 (9)	0.63 ± 0.10 (9)	
	0.90 (7)‡‡	1.09 ± 0.18 (6)	

Errors are ± s.e. with number of points in parentheses.

* Details of the weighted non-linear fit and unweighted Hill plot are given under Methods; ** References: a, Birdsall & Hulme (1976); b, Birdsall *et al.* (1976); c, Hulme *et al.* (1976). ‡ Calculated from Burgen *et al.* (1974a). § Robinson, Taylor & Young (1975). † Non-specific binding of [^{3}H]-PrBCM set to 14% (cf text and legend to Table 1). The fitted curve is shown in Figure 3. †† Racemate. ¶ Curve fitted to the filled points in Figure 2. If all points are fitted the value rises to 0.73 (23). ‡‡ 30 min preincubation.

Abbreviations: C_6TMA, hexyltrimethylammonium; C_{12}TMA, dodecyltrimethylammonium.

curves for ligands would probably be shifted but without a marked effect on the mean Hill coefficient is apparently borne out, since for the antagonists the values approached unity and gave no indication of being other than single mass action binding curves, in good agreement with the data for homogenates (Birdsall & Hulme, 1976; Birdsall *et al.*, 1976) (Table 2).

Binding of muscarinic agonists

The proportion of [^{3}H]-PrBCM binding insensitive to inhibition by the muscarinic agonists investigated was not markedly different from that for the antagonists (Table 1). However, in contrast to the antagonists, the binding of muscarinic agonists does not represent a mass action equilibrium with a single site. The inhibition curve for carbachol (Figure 1) has a mean Hill coefficient of 0.36, although as noted in the methods section the actual value serves only as an index of the deviation from a simple drug-receptor equilibrium. Even with the scatter of points there is some indication of a plateau in the carbachol inhibition curve (Figure 1), which would be anticipated whether the low value of the Hill coefficient was due to binding to two discrete sites with differing affinities or to some form of negative cooperativity. In both cases the value of Hill coefficient would vary over the length of the curve.

There is some indication that the amount of scatter observed on agonist binding curves made up of points derived from different groups of guinea-pigs at different times may not be due solely to experimental error. The inhibition of [^{3}H]-PrBCM binding by oxotremorine measured on guinea-pigs from three different batches from two suppliers is shown in Figure 2 (closed symbols). However, the values obtained from another batch of animals purchased and used in the period February–March 197 deviated systematically (open symbols, Figure 2) from the general trend and were not a property of the particular solution of oxotremorine used. A subsequent batch of animals gave points that were consistent with the original curve and consequently it has not been possible to investigate this phenomenon in more detail. Whether the effect is related to the seasonal variation that has been reported for the contractile response of guinea-pig ileum segments to acetylcholine (Weinstock & Shoham, 1974) is unknown, although we have not observed any consistent seasonal variation in the sensitivity of the contractile response to carbachol.

In view of the variable effect of oxotremorine, the best-fit value for the Hill coefficient, 0.65, neglecting the open points in Figure 2, must be treated with some reserve. An alternative fitting procedure (see Methods) gave a similar best-fit value, 0.63, but inspection of the experimental data and fitted curve (Figure 2) indicates the limitation of analysing the inhibition curves by a simple Hill equation when the Hill coefficient is not near unity. If all the points obtained (both closed and open symbols, Figure 2) are used, the best-fit value of the Hill coefficient rises to 0.73.

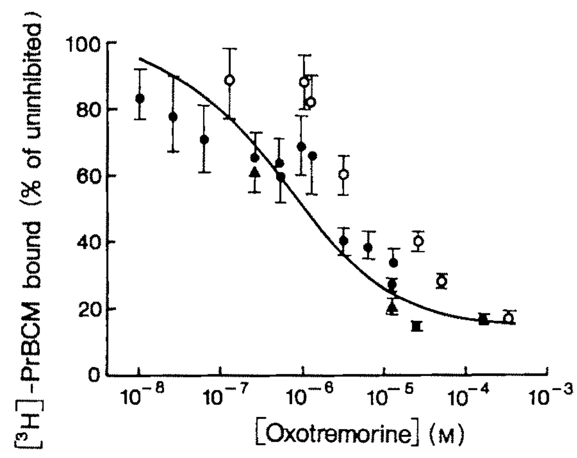

Figure 2 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by oxotremorine. The experimental conditions are described under Methods. Points obtained from one particular batch of guinea-pigs (○) differed systematically from those made on three others (●). The curve is fitted to the closed symbols (●) only. Error bars represent the 69% confidence limits. (▲) 30 min preincubation with oxotremorine.

Some confidence that the agonist inhibition curves do represent some aspect of muscarinic cholinergic action is provided by the difference in potency between the (+)- and (−)-isomers of *β*-methacholine (Figure 3). The high concentration and consequently large amount of the (−)-isomer required for the highest levels of inhibition of [^{3}H]-PrBCM binding precluded the determination of a reliable value of the Hill coefficient so that it is uncertain whether the two curves are 'parallel'. Even with the (+)-isomer the lack of a sufficient number of points at high inhibition led to a best-fit curve with only 2% non-specific binding, which in comparison with other values in Table 1 is unlikely. The potency ratio between the two isomers for production of the contractile response, measured in 3 independent experiments (2 x 2 dose assay) was 470 ± 30. This is rather larger than the reported value of 240 (Beckett, Harper, Clitherow & Lesser, 1961), but could reflect a more complete resolution of the isomers in our sample (prepared by Dr K.S. Scott). Both potency ratios were measured in the absence of a cholinesterase inhibitor. (+)-Methacholine is a substrate for acetylcholinesterase but, at least at higher concentrations of the isomer, the presence of physostigmine 10^{-6} M made no apparent difference to the level of inhibition of [^{3}H]-PrBCM binding

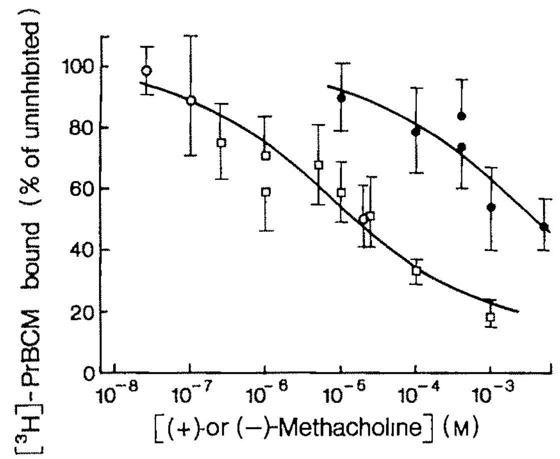

Figure 3 Comparison of (+)- and (−)-methacholine as inhibitors of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips. Error bars represent the 69% confidence limits. (□) (+)-Methacholine, no cholinesterase inhibitor present; (○) (+)-methacholine, with physostigmine 10^{-6} M present in both uninhibited and methacholine incubation vessels; (●) (−)-methacholine, no cholinesterase inhibitor present. The curve through the (−)-methacholine points has been drawn by inspection, that through the points for the (+)-isomer is calculated, but see footnote † to Table 2.

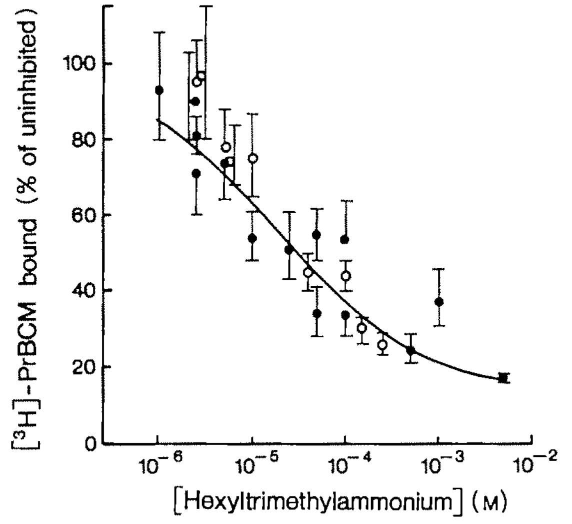

Figure 4 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding to muscle strips by hexyltrimethylammonium. The hexyltrimethylammonium was preincubated with the strips for either 1 min (●) or for 30 min (○) before the addition of [^{3}H]-PrBCM. The curve drawn has been fitted to the 1 min (●) points. Error bars represent the 69% confidence limits.

observed. The potency ratio for binding, measured at the 50% inhibition level was approximately 200, but the accuracy of this figure is clearly limited by the uncertainty of the slope and position of the curve for the (−)-isomer (Figure 3).

The IC$_{50}$ values for the inhibition of [^{3}H]-PrBCM binding to the intact muscle by muscarinic agonists were shifted to higher concentrations than those reported for homogenates (Table 1), just as for the antagonists. Only carbachol was a clear exception to this rule. However, the point of prime interest is that the Hill coefficients determined from the agonist inhibition curves are, with the reservations about oxotremorine noted above and hexyltrimethylammonium, discussed below, in reasonable agreement (Table 2) with those reported for binding to homogenates of cerebral cortex (Birdsall & Hulme, 1976; Birdsall *et al.*, 1976; Hulme *et al.*, 1976), the tissue for which the most extensive data are available. Making comparisons between two different tissues raises problems, but the evidence currently available suggests a close similarity between the biochemical properties of muscarinic receptors in cerebral cortex and in smooth muscle (see review by Birdsall & Hulme, 1976). The measure of agreement of the mean Hill coefficients for the agonists binding to the intact muscle and to cortical homogenates provides strong evidence that the low values are not diffusional or homogenization artifacts.

Binding of alkyltrimethylammonium salts

The general agreement between the Hill coefficients observed in homogenates and in the intact muscle is less clear for hexyltrimethylammonium (C$_6$TMA). There were, however, experimental difficulties with this compound, as also with nonyltrimethylammonium (C$_9$TMA), dodecyltrimethylammonium (C$_{12}$TMA) and pilocarpine, since at the concentrations necessary for higher degrees of inhibition a distinct detergent-like action was apparent. What effect this may have on the smooth muscle membrane or on the diffusional properties of the tissue is unknown. One consequence was that it was difficult to ascertain with any degree of confidence the level of inhibitor-insensitive binding and indeed with C$_9$TMA it was only feasible to determine half the inhibition curve.

Analysis of the curve for C$_6$TMA under normal conditions for agonists (Figure 4, closed symbols) is further complicated by the scatter of experimental values at concentrations of 5×10^{-5} M and above, although the weighing should to a large extent compensate for this. Even so the best-fit Hill coefficient, 0.55, was rather less than would have been expected in comparison with the value of 1.0 in cortical homogenates (Birdsall & Hulme, 1976). Increasing the period of incubation of the tissue with C$_6$TMA from 1 min, the period used for the agonists, to 30 min (Figure 4, open symbols), as employed for the antagonists, did not lead to any statistically significant increase, as judged from unweighted Hill plots, in the Hill coefficient, although the best-fit value, 0.74, was numerically greater. Increasing the incubation for C_{12}TMA from 1 to 30 min also resulted in a numerical, but again non-significant, increase in the best-fit value (Table 2). Whether for C_6TMA there is a discrepancy from the homogenate value requires further investigation, but it is interesting to note that in muscle strips exposed to distilled water, a treatment which may influence the position of agonist binding curves without apparently having any marked effect on the slope, the Hill coefficient for C_6TMA with 1 min incubation is of the order 0.6–0.7 (Elliott, Tayler & Young, unpublished observations).

Analysis of agonist binding curves as double hyperbolae

The observations presented above, taken together with the published data from broken cell preparations, make it highly unlikely that the low values of the mean Hill coefficients for strong agonists are artifactual, but give little indication of the underlying mechanism. The simplest explanation (Burgen & Hiley, 1974) is that the agonist curves do not represent binding to a single site, but to two or more with differing affinity. Only for 4 of the agonists are the data for the binding to the intact muscle strips sufficiently extensive to warrant analysis of the curves as binding to two independent sites (Tables 3 and 4). The percentage of high and low affinity sites is reasonably similar for each agonist (Table 4). There is no simple correlation with the ED_{50} for the contractile response (Table 3). In any event such correlations need to be made with care since the ED_{50} for contraction may vary widely. The ED_{50} for carbachol was calculated from measurements made at irregular intervals over a period of some 3 years, the ED_{50} varying between 3×10^{-8} M and 2.5×10^{-7} M. The measurements for oxotremorine, (+)-methacholine and methylfurmethide were made during a limited period on muscle strips from guinea-pigs which were unusually sensitive to carbachol (ED_{50} of this group, 5 experiments, $5.2 \pm 0.5 \times 10^{-8}$ M).

Discussion

The degree of agreement of the values determined for the mean Hill coefficients for muscarinic ligand binding to 'intact' longitudinal muscle strips from guinea-pig small intestine with those derived from broken cell preparations leaves little doubt that the low values of the coefficients which characterize the binding of strong agonists is not an artifact of homogenization. At the same time the general shift in the IC_{50} values underlines the problems of kinetic analysis in the intact tissue. At first sight the

Table 3 Agonist inhibition curves analysed as binding to two independent sites· dissociation constants and comparison with ED_{50} for contraction

	Dissociation constants (M) K₁	K₂	*ED_{50} contraction (M)*
Carbachol	$7.2 \pm 3.6 \times 10^{-7}$	$2.9 \pm 1.6 \times 10^{-4}$ (18)	$1.1 \pm 0.2 \times 10^{-7}$ (14)
(+)-Methacholine	$1.8 \pm 1.0 \times 10^{-7}$	$5.3 \pm 1.4 \times 10^{-5}$ (13)	$3.3 \pm 0.7 \times 10^{-8}$ (3)
Methylfurmethide	$0.95 \pm 2.2 \times 10^{-8}$	$4.2 \pm 3.9 \times 10^{-4}$ (10)	$4.9 \pm 2.4 \times 10^{-8}$ (3)
Oxotremorine*	$0.66 \pm 1.3 \times 10^{-8}$	$2.4 \pm 0.96 \times 10^{-6}$ (17)	$2.6 \pm 0.5 \times 10^{-8}$ (3)

Values for K_1 and K_2, ± estimated standard deviations (number of points), were obtained using VBOIA (see Methods). Values for ED_{50} are means ± s.e. (number of determinations).
* Only the filled points in Figure 2 were fitted.

Table 4 Agonist inhibition curves analysed as binding to two independent sites: percentage of each site

	Site (% of total binding) *High affinity*	*Low affinity*	*Non-Spec.*†
Carbachol	41.7 ± 4.0 (50)	40.9 ± 4.6 (50)	17
(+)-Methacholine	34.4 ± 4.2 (41)	50.0 ± 4.2 (59)	16
Methylfurmethide	30.7 ± 21.0 (39)	48.1 ± 19.0 (61)	21
Oxotremorine*	27.6 ± 8.9 (32)	57.8 ± 8.7 (68)	15

Values ± estimated standard deviations were obtained from VBOIA (see Methods). The figures in parentheses are the values expressed as a percentage of the receptor-specific binding.
* Only the filled points in Figure 2 were fitted. † Non-receptor binding of [^{3}H]-PrBCM 100– (site 1 + site 2).

longitudinal muscle strip has several advantages. It separates from the gut as a thin sheet of largely smooth muscle tissue, some 6–8 cells thick, with Auerbach's plexus usually adhering to the inner surface. The contractile response to agonists is practically indistinguishable from that of whole intestinal segments, while at the same time it is sufficiently 'enriched' in smooth muscle cells to give a high-proportion of receptor-specific binding in the presence of low concentrations of [^{3}H]-PrBCM. It seems very probable however that diffusional problems remain.

The question of the presence or otherwise of a 'biophase' barrier in this tissue in the onset and offset of antagonist action has been discussed by earlier authors (Rang, 1966; Thron & Waud, 1968) and more recently Roberts & Stephenson (1976) have shown that the kinetics of antagonist action are not satisfactorily described by a simple interaction-limiting model. The sequential binding sites model of Paton & Rang (1965), which largely accounts for the anomalous kinetics of [^{3}H]-atropine uptake and release from longitudinal muscle strips, can be viewed as another expression of the diffusional problem, since it may be expected with some confidence that the process of diffusion in the inter-cellular channels will be strongly influenced by reversible interactions with numerous low affinity binding sites. We have also concluded from the increase in the rate of binding [^{3}H]-PrBCM in homogenates as compared with intact preparations that the presence of a diffusion barrier is very probable (Taylor, Cuthbert & Young, 1975) and the displacement of inhibition curves to higher inhibitor concentrations is in accord with this. However, the extent of the difference between IC_{50} values in homogenates and in intact muscle is variable and it is by no means certain that it can be accounted for entirely by the simple considerations applied here (cf. Methods section). For particular ligands other factors are clearly involved. The discrepancy for benzhexol is notably large while the IC_{50} for carbachol is in curiously good agreement with broken cell systems. Indeed, earlier measurements with carbachol (Young, 1974) indicated a leftward shift of the % of uninhibited uptake *versus* [carbachol] curve without any apparent change in slope as the period of incubation with [^{3}H]-PrBCM was reduced from 10 to 5 min, which might be anticipated on the simple theory developed under Methods, without the necessity of invoking ongoing desensitization. These particular cases require further examination and it would be of particular interest to know whether the changes in the ED_{50} values for the contractile response to carbachol are mirrored by corresponding changes in the position of the carbachol binding curve.

Although little doubt can remain that the binding of strong agonists cannot be described by a single mass-action equilibrium, the data presented here allow few conclusions to be drawn of the mechanism by which the flattened binding curves result. The two main possibilities open are that the curves represent binding to two or more independent sites or, alternatively, that the low Hill coefficients reflect the kinetic mechanism operating. The evidence currently available is consistent with the presence of two independent sites (Birdsall *et al.*, 1976; Hulme *et al.*, 1976). There is little indication of any negative cooperativity of muscarinic agonist binding and Birdsall *et al.* (1976) have demonstrated that occlusion of some 90% of muscarinic receptor sites in cortical homogenates by treatment with an irreversible antagonist (PrBCM) did not alter the mean Hill slope for carbachol binding to the remaining 10%. Similarly a suggestion that desensitization might be involved, on the basis of the effect of a large dose of carbachol of increasing the Hill coefficient of the binding curve for carbachol, measured immediately after a brief wash, in intact muscle strips (Young, 1974), now seems unlikely since no similar effect has been found in cortical homogenates (Birdsall & Hulme, 1976). The mechanism of the carbachol effect in intact muscle remains to be established.

Of reports in the literature of more than one site for muscarinic agonist action in the longitudinal muscle strip preparation (Burgen & Spero, 1968; Bolton, 1975; Kilbinger, 1975; Kilbinger & Wagner, 1975) that of Burgen & Spero (1968), who compared the effects of muscarinic agonists on causing contraction and, at higher concentrations, on promoting the efflux of K^+ and Rb^+, provides the most extensive data for comparison with agonist binding. However, there is no apparent correlation between the affinities of agonists for their two sites and the dissociation constants for binding, deduced from analysis of curves as double hyperbolae (Table 3), although presumably the Rb^+ efflux site will have been labelled by [^{3}H]-PrBCM since the affinity of antagonists for this site was the same as against contraction (Burgen & Spero, 1968). It is interesting to note, however, that the one antagonist which appeared to have different affinities against efflux and contraction was benzhexol and although the Hill coefficient for benzhexol binding is unity there is an unusually large difference between IC_{50} values in strips and in homogenates.

The weight of the evidence rests with two independent sites, but there is little indication what their function might be. It is, however, of particular interest that the curve for the stimulation of phosphatidylinositol turnover in longitudinal muscle strips (Jafferji & Michell, 1976) corresponds closely to the binding curve for carbachol. If phosphatidylinositol breakdown is intrinsic to the mechanisms of muscarinic receptor systems, as has been suggested (Michell, 1975), then both binding sites would seem to be involved with the production of a response.

We thank the Science Research Council for financial support (B/RG/5409).

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(Received December 20, 1976.
Revised March 29, 1977.)

Br. J. Pharmac. (1977), **61**, 199–212

THE EFFECTS OF INTRAVENOUS ANAESTHETICS ON THE CARDIOVASCULAR SYSTEM OF THE RABBIT

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1 A pithed rabbit preparation is described that allows selective stimulation of the vertebral outflows.

2 The responses to stimulation of sympathetic vasopressor fibres were blocked by hexamethonium and phentolamine but potentiated by cocaine, whereas the responses to stimulation of cardioaccelerator fibres were blocked by propranolol.

3 Ketamine, althesin and pentobarbitone enhanced the effects of noradrenaline and attenuated the effects of sympathetic nerve stimulation. Thiopentone enhanced the effects of both noradrenaline and sympathetic nerve stimulation.

4 In pithed rabbits a transient, dose-related cardiovascular depression was produced by each agent irrespective of whether vasomotor tone was present whereas in decerebrate rabbits the corresponding cardiovascular depression was longer lasting.

5 It is concluded that the cardiovascular depression produced by intravenous anaesthetics in intact rabbits is due to a combination of central and peripheral effects.

Introduction

Anaesthetic agents can affect the cardiovascular system by eliciting effects at several sites simultaneously to produce a net effect which is often difficult to interpret in the intact animal (Price, 1960). These may include the inhibition of reflexes as well as direct central or peripheral actions.

In a recent study with several intravenous anaesthetics (McGrath, Mackenzie & Millar, 1975a; Mackenzie, McGrath, Tetrault & Millar, 1976) in decerebrate or pentobarbitone-anaesthetized rabbits the effects on the cardiovascular system could be correlated with changes in central sympathetic activity. However, the relative contributions of central and peripheral actions to the net effects could not be distinguished. We have, therefore, compared the effects of the intravenous agents thiopentone, althesin and ketamine on the cardiovascular system of the pithed rabbit with those on the decerebrate rabbit in order to identify the peripheral components of their actions and as a corollary to define more closely the peripheral consequences of their central actions.

In this study the pithing rod was introduced via a trephine hole in the skull instead of via the orbit of the eye. The latter mode of entry is not possible in the rabbit due to the anatomy of the skull. The technique of Gillespie MacLaren & Pollock (1970) for pithed rats and cats was also extended by making the additional measurements of cardiac output and left ventricular pressure.

The pithed rabbit preparation was first examined with respect to the effects of stimulation of the autonomic outflow at different levels and to the influence of specific antagonists on these responses.

Secondly the effects of the intravenous anaesthetics althesin, thiopentone, pentobarbitone and ketamine were tested on the cardiovascular responses of the pithed rabbit to noradrenaline and to sympathetic nerve stimulation. It was of interest to compare the purely peripheral effects of these agents since in clinical use (Domino, Chodoff & Corssen, 1965; Clarke, Montgomery, Dundee & Bovill, 1971; Coleman, Downing, Leary, Moyes & Styles, 1972) and in the conscious rabbit (McGrath *et al.*, 1975a; Mackenzie *et al.*, 1976) they produce contrasting effects, althesin behaving rather like the barbiturates in depressing the cardiovascular system, while ketamine elevates both blood pressure and heart rate.

Finally, the sympathetic outflow in the pithed rabbit was continuously stimulated in order to elevate artificially the peripheral resistance to the level found in decerebrate rabbits and a comparison was made of the cardiovascular actions of the intravenous anaesthetics on the two preparations.

A preliminary report of some of these results has been published (McGrath & Mackenzie, 1976).

Methods

Adult male New Zealand white rabbits (3.0–3.5 kg) were anaesthetized with 3.0% halothane in 100% O_2. The trachea was intubated and anaesthesia continued with 2.5% halothane. The left carotid artery was cannulated with a pair of concentric cannulae, each connected to a pressure transducer. The inner cannula was introduced until its tip lay in the left ventricle as measured by the change in the pressure pulse, and the outer one left with its tip in the carotid artery approximately 10 mm from the aorta. A thermistor probe was introduced into the right carotid artery until its tip lay in midstream in the aorta and a cannula was introduced into the right jugular vein for administration of drugs and of cold saline (0.9% w/v NaCl solution) for measurement of cardiac output by the cold dilution method (Fegler, 1954).

The rabbit was then turned over into the prone position, its head held rigid in a metal head holder and a 13 mm trephine hole drilled in the parietal area of its skull. This hole was subsequently enlarged with bone nibblers so that both sides of the parietal skull were removed. After increasing the halothane concentration to 3%, mid-collicular decerebration was performed by suction. With both carotid arteries effectively occluded and the arterial pressure lowered due to halothane, bleeding was minimal. Halothane administration was then terminated, gallamine (1 mg/kg i.v.) given (to prevent muscle twitching due to stimulation of ventral root motor fibres in the subsequent experiment) and mechanical ventilation (Harvard Instruments Ventilator, Model 613) with 100% O_2 employed to maintain the end tidal CO_2 at 4% (Beckman LB2 Medical Gas Analyser).

From this point the animals were treated in one of three ways.

(1) *Pithed rabbit*

The rabbit was fully pithed via the trephine hole. The pithing rod consisted of a stainless steel rod (2 mm in diameter) which was covered with a teflon sheath (o.d. 3.6 mm) except for 12 mm at its tip.

The autonomic outflows were stimulated using as electrodes the unshielded tip of the pithing rod and an indifferent electrode of silver wire placed subcutaneously dorsal and parallel to the cervical vertebrae (stimulation parameters: Devices Isolated Output Stage, 1 ms pulses, supramaximal voltage (nominal 70–100 V), frequencies indicated in text). The position of the electrode tip was gauged by its distance from the tentorium cerebelli, visible through the trephine hole. This position relative to the vertebrae was then checked by X-ray at the end of the experiment.

Where appropriate, continuous stimulation at T8 for 7–8 min produced a maintained elevation of arterial pressure. Test drugs were administered when the elevated arterial pressure had reached a plateau for at least 2 minutes. In each experiment an initial control period of such stimulation was carried out to verify that the pressor response would be maintained in the absence of drugs.

(2) *Decerebrate rabbits subsequently pithed*

One group of six rabbits were allowed 1 h to recover from the anaesthetic following decerebration before pithing in order that various cardiovascular parameters could be measured before and after pithing in the same rabbits (see Table 3).

(3) *Decerebrate rabbits*

In this group the rabbits were allowed to recover from the anaesthetic and pithing was omitted. The earlier placement of carotid cannulae was also omitted, arterial pressure being recorded from a cannula advanced into the abdominal aorta via the femoral artery.

Arterial pressure, left ventricular pressure, the differential of left ventricular pressure (dP/dt), the heart rate extracted from left ventricular pressure, and end tidal CO_2 were all displayed on a Devices M19 6-channel pen recorder.

Throughout each experiment arterial blood samples were taken at least every 60 min for analysis of $P\text{O}_2$, $P\text{CO}_2$ and pH (IL Models 213 and 227). Base excess was calculated and, where necessary, the appropriate amount of sodium bicarbonate was given to correct acidosis.

Rectal temperature was maintained at 38.0 ± 0.5°C with a homoeothermic blanket (C.F. Palmer).

Drugs were administered as a bolus injection via the femoral vein in the case of decerebrate and the jugular vein in the case of pithed rabbits. Preliminary experiments indicated that the choice of vein made no material difference to the responses under observation so the jugular vein was left intact in the decerebrate rabbits.

The doses of althesin, ketamine and thipentone used in this study were selected as those having slightly sub-anaesthetic, low-anaesthetic and moderate anaesthetic effects in rabbits and falling within the range used in previous studies on the baroreceptor reflex (McGrath, Mackenzie & Millar, 1975b; Mackenzie *et al.*, 1976). The dose of pentobarbitone used was that which maintained anaesthesia in these latter studies where the rabbits were not decerebrate.

At least 40 min was left between consecutive injections of the agents to allow full recovery. In the case of pentobarbitone this was extended to at least 70 minutes.

Statistical analysis was performed using Student's *t* test.

Drugs

The following drugs were used: althesin (Glaxo Laboratories: preparation composed of alphaxalone 0.9% and alphadolone acetate 0.3% w/v in a solvent of polyoxyethylated castor oil 20% in saline; doses are stated in ml/kg), cocaine hydrochloride, hexamethonium bromide (Sigma), halothane (Fluothane, ICI), gallamine triethiodide (Flaxedil, May & Baker), isoprenaline sulphate (Boots), ketamine hydrochloride (Parke-Davis), (−)-noradrenaline bitartrate (Sigma), pentobarbitone sodium (Nembutal, Abbott), phentolamine mesylate (Rogitine, CIBA), (±)-propranolol hydrochloride (Sigma) and thiopentone sodium (Intraval Sodium, May & Baker).

Results

Characteristics of the pithed rabbit preparation

Maintenance of the preparation. Following pithing, the arterial pressure rose to a mean value in excess of 150 mmHg as a result of damage discharge from the sympathetic outflow, but then fell steadily over a period of 5–10 min to a resting level of 54.0 ± 2.4 mmHg systolic, 27.4 ± 1.7 mmHg diastolic.

The resting levels of arterial pressure, heart rate and cardiac output (Table 2) were maintained consistently throughout an experimental period of approximately 6 h at which time the experiments were terminated by stopping the respirator.

With the respirator set to give an end tidal CO_2 of approximately 4% (rate 25–30 per min, stroke volume approximately 50 ml), it was not normally necessary to adjust this further. The tendency towards acidosis varied between animals, but on average 0.125 mEq kg^{-1} h^{-1} of sodium bicarbonate was given.

Responses to stimulation of the autonomic outflow at different levels. Since the dimensions of the vertebrae were similar throughout the group of rabbits used, the distance of the tip of the pithing rod electrode from the tentorium cerebelli was a reliable guide to the electrode's anatomical position. Table 1 relates the anatomical position of the electrode to this distance.

Figure 1 (a, b and c) shows the responses produced by stimulating the autonomic outflows with a fixed train of stimuli at successive 20 mm intervals along the vertebral canal in a rabbit. In the upper lumbar region small rises in arterial pressure were produced which increased in size as the electrode was moved rostrally into the thoracic region, reaching a maximum at T4-5 (14 cm from reference point). The heart rate began to increase at T7 (16 cm from the reference point). Rostral movement to T3 (12 cm from reference point) produced further increase in the heart rate. At T3, although the arterial pressure response was less than at T4-5, the pulse pressure increased, indicating a pressure rise due to an increase in cardiac output rather than an increase in peripheral resistance (Figure 1d).

In addition, at the position where an increase in heart rate occurred, a proportional increase in arterial pulse pressure was found. It was thus concluded that with the electrode in the region sacral to T7, pressor responses that were produced were due solely to stimulation of vasopressor fibres, at T3 they were mainly due to stimulation of sympathetic fibres to the heart, and at intermediate positions they were due to both. In subsequent experiments, therefore, 2 positions were selected as suitable for observing the effects of blocking drugs, T8 (17 cm) and T3 (12 cm) as the optimum examples for vasopressor and cardioaccelerator stimulation respectively.

Effect of frequency of stimulation on autonomic responses. The effects of stimulation of the autonomic outflow at T8 at different frequencies are shown in Figure 1. Frequencies from 1 Hz to 20 Hz produced frequency-related rises in arterial pressure without an increase in heart rate. At frequencies up to and including 10 Hz the responses were well maintained over the 2 min stimulation period, while at 20 Hz the response declined slowly after reaching its peak. The increase in arterial pressure at 5 Hz could be maintained at a plateau level for up to 12 min of continuous stimulation. There was also a frequency-related increase in positive left ventricular dP/dt_{max} and in left ventricular end-diastolic pressure

Table 1 Relationship between the length of the pithing rod electrode sacral to the tentorium cerebelli and the position of the unshielded electrode at the tip of the pithing rod

Length of rod (cm)	28	26	24	22	20	18	17	16	14	12
Vertebral outflow adjacent to the electrode	L3	L2	L1	T11	T10	T9	T8	T7	T5,4	T3

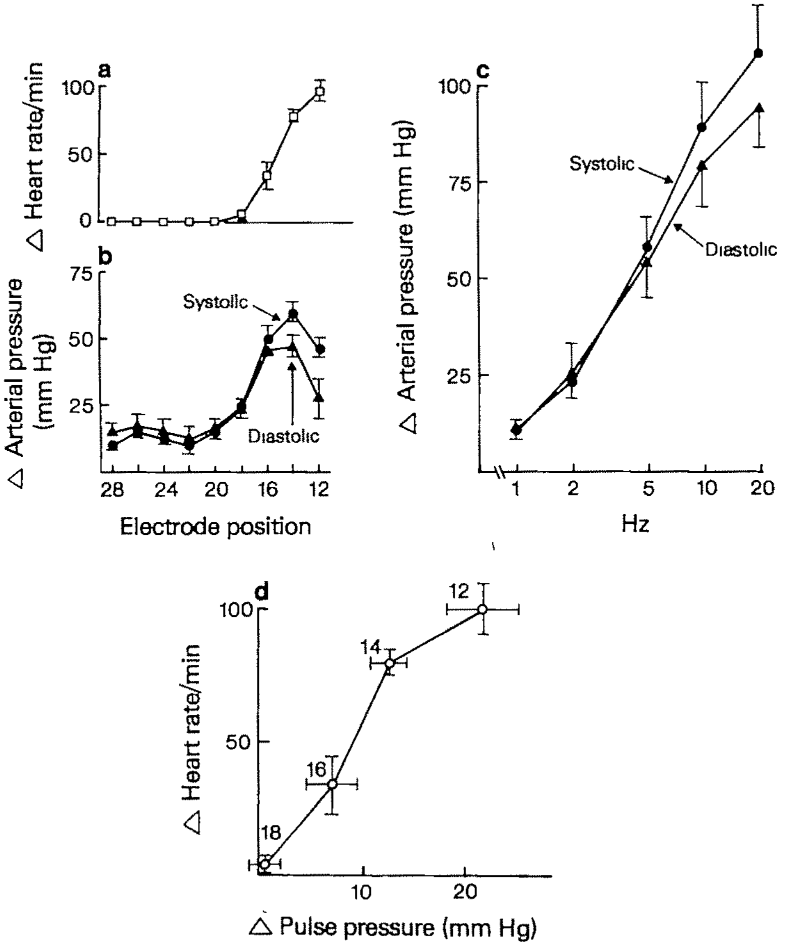

Figure 1 The increase in (a) heart rate and (b) arterial pressure (systolic, diastolic) resulting from electrical stimulation of the sympathetic outflow with the electrode at various positions along the vertebral canal relative to the reference point (see Table 1). Stimulation parameters 10 Hz, 20 s period. Points represent mean for 6 rabbits. Vertical lines show s.e. mean. Note that although an elevation of arterial pressure is elicited by stimulation at positions between L3 and T7 (28–20 cm), there is no effect on heart rate. (c) The effect of stimulation of the sympathetic outflow at T8 (17 cm) at various frequencies on the stystolic (●) and diastolic (▲) blood pressure. Responses are the maximum increases obtained during continuous stimulation at each frequency. Points represent mean for the same 6 rabbits as in (a) and (b). Vertical lines show s.e. mean. (d) Relationship between the change in heart rate and the change in arterial pulse pressure. Results are the mean for the same six rabbits as in (a) and (b). Horizontal and vertical lines show s.e. mean. Vertebral position of the electrode is indicated on the graph.

(2.4 ± 0.3 mmHg, $n=5$) which indicates that in this situation the non-innervated heart was responding to the increased peripheral resistance by increasing its force of contraction.

The maximum increases in systolic and diastolic arterial pressure to continuous stimulation at different frequencies from 6 experiments are shown in Figure 1c. In view of the responses shown in Figure 1c, a standard stimulation period of 10 Hz for 20 s was selected as capable of producing a submaximal response which could be either increased or decreased by the action of drugs.

Continuous stimulation of the sympathetic outflow. Stimulation at T8 (5 Hz) for a period of up to 12 min produced a maintained elevation of arterial pressure due to an increased peripheral resistance. Left ventricular dP/dt_{max} and cardiac output were also increased, whereas heart rate was unaffected (Table 2).

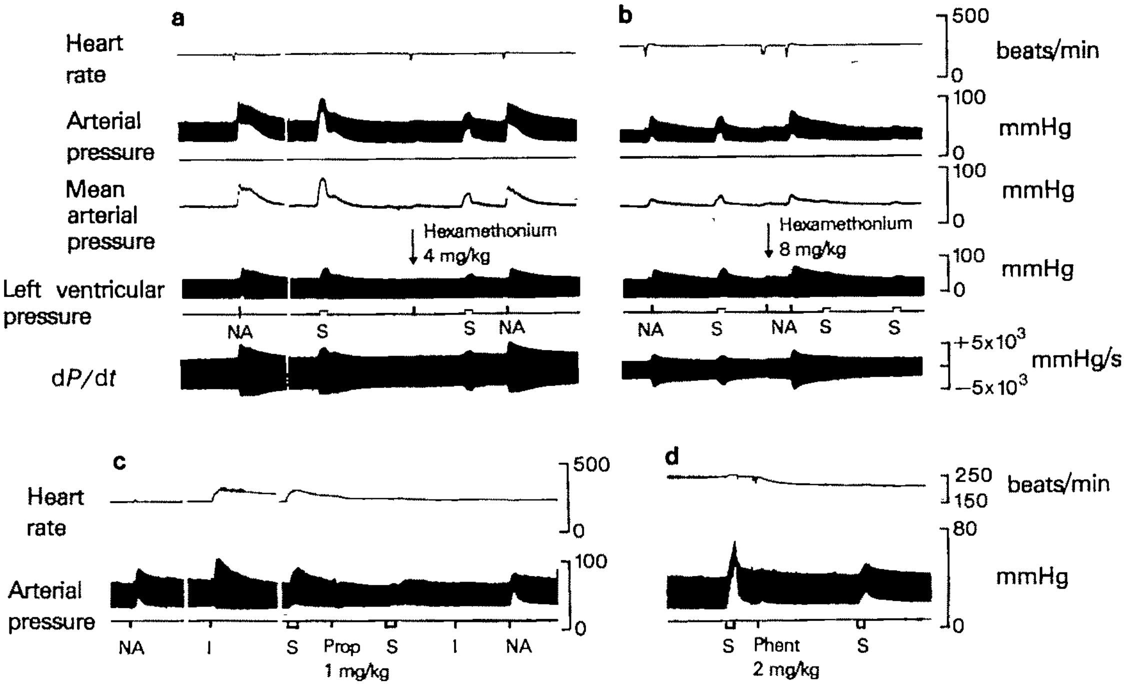

Figure 2 The effects of antagonist drugs on cardiovascular responses in the pithed rabbit. The effects of 2 doses of hexamethonium, in (a) 4 mg/kg, and (b) 8 mg/kg, on the responses to electrical stimulation of sympathetic outflow at T8 (S, 10 Hz, 20 s) and to noradrenaline (NA, 0.3 μg/kg). Note that although the response to electrical stimulation is greatly reduced, the response to noradrenaline is unaffected. (c) Effects of propranolol (Prop, 1 mg/kg) on responses to stimulation of the sympathetic outflow (S, T3, 10 Hz, 20 s), noradrenaline (NA, 0.5 μg) and isoprenaline (I, 1 μg). (d) Effects of phentolamine (Phent, 2 mg/kg) on the response to stimulation of the sympathetic outflow (S, T8, 10 Hz, 20 s). Note that β-adrenoceptor antagonism by propranolol abolishes the increase in heart rate produced by isoprenaline and electrical stimulation. The vasopressor component of electrical stimulation is greatly reduced by the α-adrenoceptor antagonist phentolamine. Time marker in minutes.

Table 2 Absolute levels of cardiovascular parameters in pithed rabbits

	Control	*Continuous stimulation*	n	P*
Cardiac output (ml/min)	327±22	408±22	15	<0.001
Stroke volume (ml)	1.48±0.10	1.86±0.11	15	<0.05
Heart rate (beats/min)	215±5	221±6	22	<0.5
Peripheral resistance (mmHg ml^{-1} min)	0.152±0.012	0.228±0.022	15	<0.001
d*P*/d*t* +ve *max* (mmHg/s)	1080±77	1760±144	18	<0.001
Mean arterial pressure (mm Hg)	39.4±1.9	75.1±4.6	22	<0.001

Control—resting levels. Stimulation—plateau levels in the same rabbits 3 min after commencing continuous stimulation (T8, 5 Hz). Each parameter is expressed as a mean ± s.e. mean.

* *P* values indicate the significance levels of the difference between the 'control' and 'continuous stimulation' group for each parameter calculated by paired *t* test.

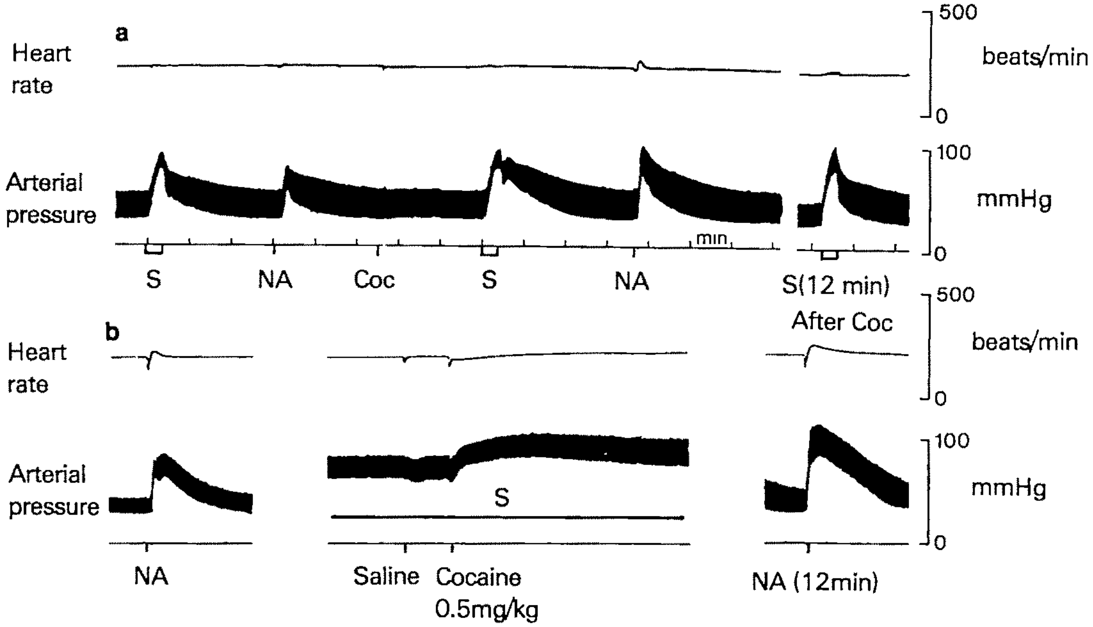

Figure 3 The potentiation by cocaine (Coc, 0.5 μg/kg) of the responses due (a) to stimulation of the sympathetic outflow (S, T8, 10 Hz, 20 s), and noradrenaline (NA, 0.3 μg/kg), and (b) to continuous stimulation at bar (T8, 5 Hz) in a subsequent experiment. Note that whilst cocaine potentiates the effects of continuous stimulation, it does not affect the resting arterial pressure in the absence of such stimulation.

Effects of blocking drugs. The arterial pressure response to stimulation at T8 was inhibited by hexamethonium (4 mg/kg and 8 mg/kg) whereas the response to an intravenous dose of noradrenaline (0.3 μg/kg), producing a similar pressor response, was not (Figure 2a, b). It was necessary to employ the rather high dose of hexamethonium (8 mg/kg) to abolish the pressor response to sympathetic stimulation (Figure 2b), and an even higher dose (16 mg/kg) to abolish the response to stimulation at T3 of the cardio-accelerator nerves. The blockade was nevertheless specific in that the responses to the respective agonists, noradrenaline and isoprenaline, were unaffected by these doses.

Isoprenaline (1 μg) produced a rise in heart rate and systolic arterial pressure similar to that obtained by stimulation at T3. The heart rate responses to isoprenaline and to sympathetic nerve stimulation were abolished by propranolol (1 mg/kg). Noradrenaline (0.3 μg/kg) produced a small transient rise in heart rate as well as a vasopressor response. Propranolol (1 mg/kg) abolished this latter heart rate response but not the corresponding vasopressor response (Figure 2c).

Phentolamine (2 mg/kg) antagonized the arterial pressor response to stimulation at T8 (Figure 2d) and the arterial pressor response to noradrenaline (0.3 μg/kg).

Effects of cocaine. Cocaine (0.5 mg/kg) did not itself alter arterial pressure or heart rate but increased the arterial pressure responses produced by stimulation at T8 and by noradrenaline (0.3 μg/kg) and the heart rate response produced by noradrenaline (0.3 μg/kg) (Figure 3a). In another experiment, cocaine (0.5 mg/kg) was given during a period of continuous stimulation at 5 Hz (T6–7) (Figure 3b, centre panel). In this latter situation, cocaine elevated arterial pressure. After stopping electrical stimulation, the arterial pressure returned to control level and the responses of both arterial pressure and heart rate to noradrenaline (0.3 μg/kg) were potentiated (Figure 3b).

Effects of intravenous anaesthetics in the pithed rabbit

Effects on responses to trains of vasopressor stimulation or to noradrenaline

Althesin and ketamine. The effects of 3 doses of each of the intravenous anaesthetics were tested against the pressor responses to an intravenous injection of noradrenaline (0.3 μg/kg) or to stimulation of the sympathetic outflows at T8 (10 Hz for 20 seconds).

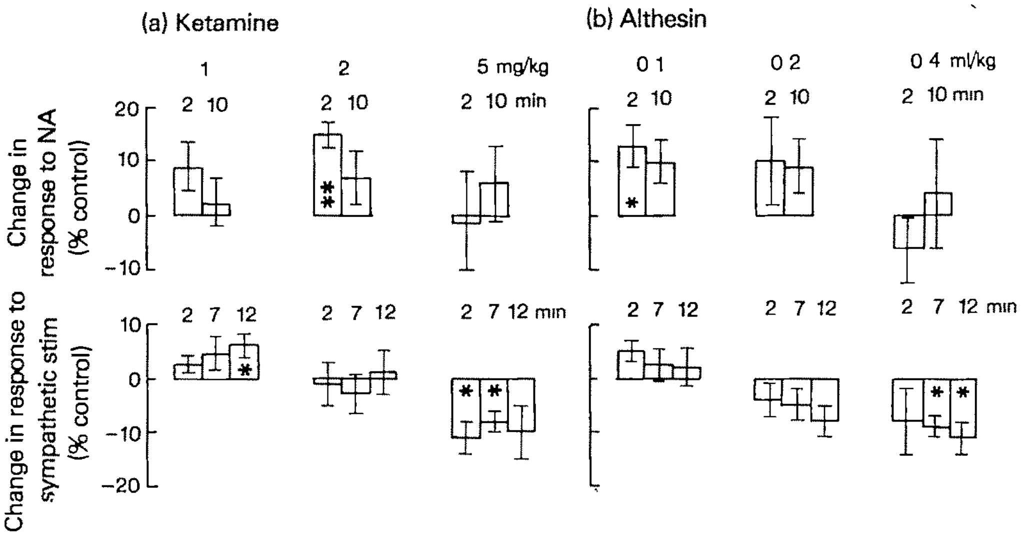

Figure 4 The effects of (a) ketamine and (b) althesin on the responses of the mean arterial pressure of the pithed rabbit to noradrenaline (NA, 1 μg) or to sympathetic nerve stimulation (T8, 10 Hz, 20 seconds). Responses at the times after injection indicated above the columns were expressed as a percentage of pre-drug control responses. The columns represent the mean change from control, vertical lines show s.e. mean ($n=5$). The doses of anaesthetic tested were, from left to right, (a) ketamine, 1, 2 and 5 mg/kg, (b) althesin 0.1, 0.2 and 0.4 ml/kg.

Asterisks denote significance of statistical comparison with controls by Student's t test. $^{*}0.05>P>0.01$; $^{**}0.01>P$.

The effects of the two agents were similar. Both slightly potentiated pressor responses to noradrenaline at the lower doses but failed to do so at the highest doses. Both also produced a small potentiation of the response to sympathetic nerve stimulation at the lowest dose and depressed these responses at the highest dose with the effect of the intermediate dose taking an intermediate position (Figure 4).

Pentobarbitone and thiopentone. In 4 out of 5 rabbits, pentobarbitone (5 mg/kg) enhanced the effects of noradrenaline and attenuated the effects of sympathetic nerve stimulation in a similar manner to the higher doses of ketamine and althesin. This effect was not, however, statistically significant (Figure 5b). The solvent in which pentobarbitone was dissolved was given in the same volume dosage to three rabbits but had no effect on any of the responses monitored.

Thiopentone enhanced the effects of both noradrenaline (significantly at 4 mg/kg) and sympathetic nerve stimulation (significantly at 2 mg/kg). The highest dose of thiopentone (8 mg/kg) had no significant effect (Figure 5a).

Direct effects on the cardiovascular system. The effects of intravenous anaesthetics were assessed in pithed rabbits first in the absence of any nerve stimulation and then during continuous stimulation of the lower thoracic vertebral outflows.

In the absence of sympathetic nerve stimulation each of the intravenous agents tested produced an immediate but short-lived depression of the cardiovascular system which was maximal within 30 s post-injection with full recovery occurring within 2–3 min (Figures 6a, 7a, 8a, 9a).

With each agent this cardiovascular depression was dose-related and the degrees of depression produced by the small, intermediate and large doses were comparable between agents.

In each case the heart rate, left ventricular systolic pressure and myocardial contractility (dP/dt_{max}) fell on a similar time course to the fall in arterial pressure (Figures 6a, 7a, 8a, 9a).

During continuous nerve stimulation the four intravenous anaesthetic agents tested produced cardiovascular depression on a similar time scale to that found in its absence. The extent of depression of arterial and ventricular pressure and of dP/dt was larger in absolute terms than in the absence of stimulation (Figure 10) and in the case of all but althesin was greater even when expressed as a percentage of pre-injection levels (Figures 6b, 7b, 8b, 9b). The falls in heart rate were similar to those found in the absence of sympathetic stimulation (Figures 6b,

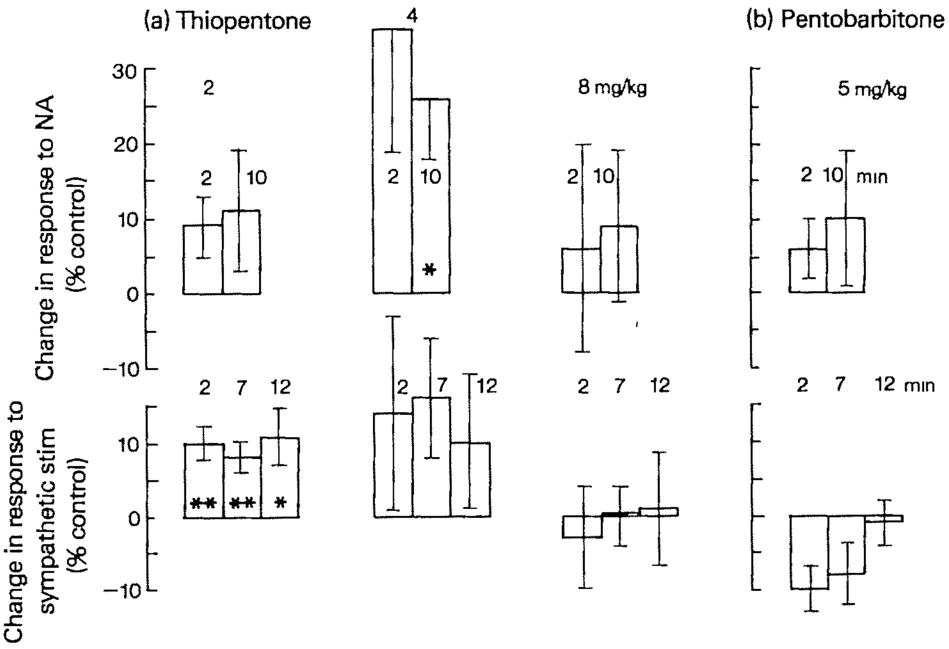

Figure 5 The effects of (a) thiopentone and (b) pentobarbitone on the responses of the mean arterial pressure of the pithed rabbit to noradrenaline (NA, 1 μg) or to sympathetic nerve stimulation (T8, 10 Hz, 20 seconds). Responses at the times after injection indicated above the columns were expressed as a percentage of pre-drug control responses. The columns represent the mean change from control; vertical lines show s.e. mean ($n=5$). The doses of anaesthetic tested were, from left to right, (a) thiopentone 2, 4 and 8 mg/kg, (b) pentobarbitone 5 mg/kg.
Asterisks denote significance of statistical comparison with controls by Student's t test. *$0.05>P>0.01$; **$0.01>P$.

Table 3 Comparison of several cardiovascular parameters in a group of rabbits (a) when decerebrate, (b) following pithing, (c) following pithing and during continuous sympathetic vasopressor nerve stimulation (T8, 5 Hz)

	(a) *Pre-pithed*	*(b)* *Pithed*	*(c)* *Continuous stimulation*
Cardiac output (ml/min)	491 ± 66	301 ± 30	387 ± 29
Stroke volume (ml)	1.59 ± 0.18	1.48 ± 0.19	1.90 ± 0.10
Heart rate (beats/min)	307 ± 15	208 ± 11	208 ± 10
Peripheral resistance (mmHg ml^{-1} min)	0.299 ± 0.024	0.151 ± 0.029	0.259 ± 0.05
Diastolic arterial pressure (mmHg)	86.1 ± 6.1	28.3 ± 3.1	59.0 ± 7.3
Systolic arterial pressure (mmHg)	121.2 ± 5.2	58.2 ± 4.5	89.0 ± 7.3
Mean arterial pressure (mmHg)	103.4 ± 5.7	40 ± 3.6	71.3 ± 9.7
Left ventricular end diastolic pressure (mmHg)	10.2 ± 2.5	−1 ± 2	2.2 ± 1.8
dP/dt +ve *max* mmHg/s	3720 ± 420	1296 ± 91	1930 ± 163

Values are means ± s.e. mean ($n=6$).

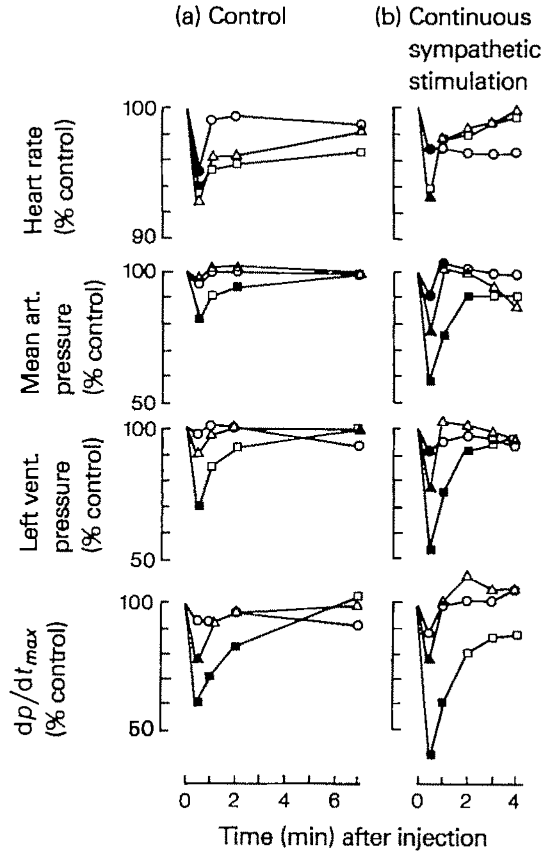

Figure 6 The effects of thiopentone on the cardiovascular system of the pithed rabbit. (a) In the absence of sympathetic nerve stimulation; (b) in the presence of continuous stimulation of the sympathetic outflow at T8 (5 Hz). Thiopentone doses: (○) 2 mg/kg, (△) 4 mg/kg; (□) 8 mg/kg. Each parameter is expressed as a percentage of pre-injection levels. The first point on each graph represents the maximum effect, which occurred between 15 and 30 s after injection. Filled symbols indicate points that are significantly different from pre-injection control levels, i.e. $P < 0.05$, $n = 5$.

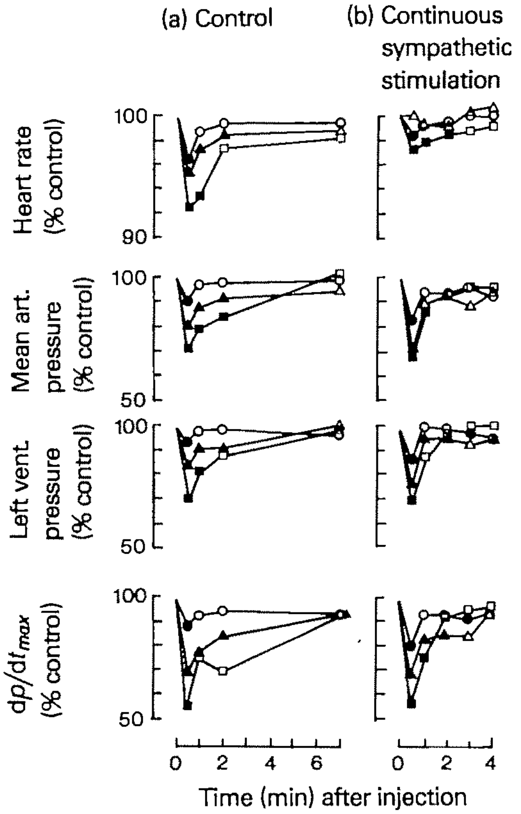

Figure 7 The effects of althesin on the cardiovascular system of the pithed rabbit. (a) In the absence of sympathetic nerve stimulation; (b) in the presence of continuous stimulation of the sympathetic outflow at T8 (5 Hz). Althesin doses: (○) 0.1 ml/kg, (△) 0.2 ml/kg, (□) 0.4 ml/kg. Each parameter is expressed as a percentage of pre-injection levels. The first point on each graph represents the maximum effect, which occurred between 15 and 30 s after injection. Filled symbols indicate points that are significantly different from pre-injection control levels, i.e. $P < 0.05$, $n = 5$.

8b, 9b, 11) with again the exception of althesin which produced smaller falls in heart rate in the presence of sympathetic vasopressor stimulation (Figures 7b and 11).

Comparison of pithed and decerebrate rabbits

Table 3 compares the resting values of various cardiovascular parameters in decerebrate rabbits before and after pithing. The peripheral resistance of the decerebrate rabbits was comparable with those in the pithed rabbits when the sympathetic outflows of the latter were continuously stimulated. The arterial pressure, heart rate, d*P*/d*t* and cardiac output were however greater in the decerebrate preparation.

When intravenous anaesthetics were administered several differences emerged between the rabbit preparations. The most striking difference was on arterial pressure. In the decerebrate rabbits the fall in arterial pressure was greater in magnitude and duration than that obtained in the pithed rabbits. In the pithed rabbits whose sympathetic nerves were continuously stimulated, and whose initial arterial pressures were relatively close to those in the decerebrate rabbits,

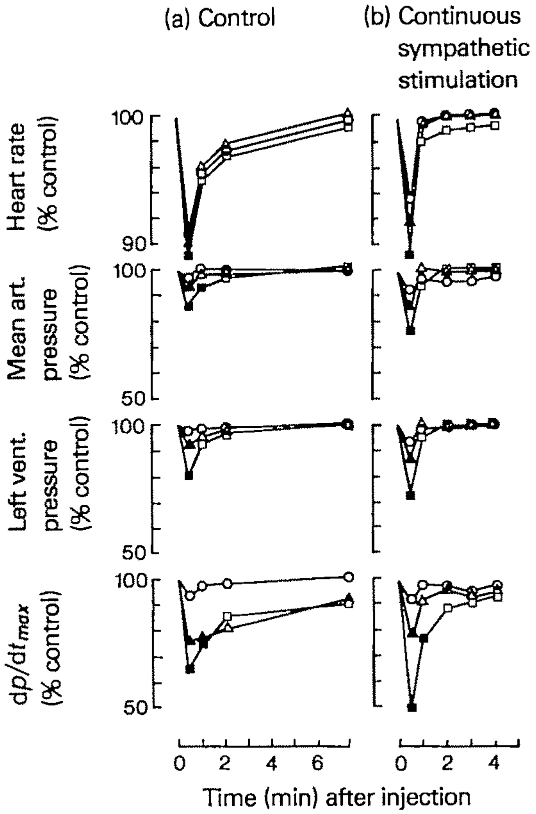

Figure 8 The effects of ketamine on the cardiovascular system of the pithed rabbit. (a) In the absence of sympathetic nerve stimulation; (b) in the presence of continuous stimulation of the sympathetic outflow at T8 (5 Hz). Ketamine doses: (○) 0.1 mg/kg, (△) 0.2 mg/kg, (□) 0.5 mg/kg. Each parameter is expressed as a percentage of pre-injection levels. The first point on each graph represents the maximum effect, which occurred between 15 and 30 s after injection. Filled symbols indicate points that are significantly different from pre-injection control levels, i.e. $P<0.05$, $n=5$.

recovery was complete within 2–3 min whereas in the decerebrate rabbits depression was still present at this time.

The falls in heart rate produced by the intravenous agents followed a similar time scale on decerebrate and pithed rabbits except in the case of ketamine which produced no significant changes in the decerebrate preparation (Figure 11).

Discussion

The pithed rabbit provides a preparation on which to examine the peripheral cardiovascular effects of

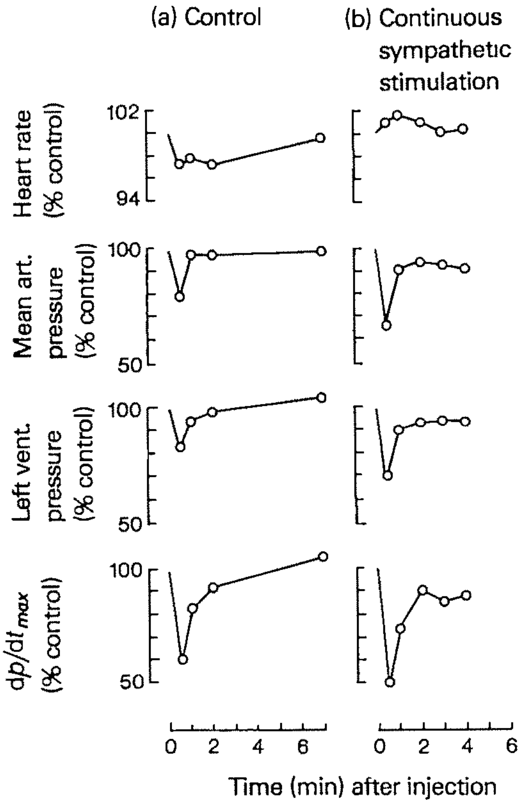

Figure 9 The effects of pentobarbitone (5 mg/kg) on the cardiovascular system of the pithed rabbit. (a) In the absence of sympathetic nerve stimulation; (b) in the presence of continuous stimulation of the sympathetic outflow at T8 (5 Hz). Each parameter is expressed as a percentage of pre-injection levels. The first point on each graph represents the maximum effect, which occurred between 15 and 30 s after injection.

autonomic nerve stimulation or drugs and has been employed here to assess the effects of intravenous anaesthetics on peripheral sympathetic nerve responses.

Stimulation of the vertebral outflows with the pithing rod allows selective stimulation of the cardiac sympathetic nerves at T3 or the vasopressor sympathetic nerves at T8. One major difference between the rabbit and the rat or cat (Gillespie *et al.*, 1970) is that no large adrenal pressor or cardio-accelerator response was encountered despite exploration throughout the thoracolumbar region. No explanation of this discrepancy is immediately apparent. The adrenals of the rabbit contain an extremely high proportion of adrenaline (West, 1955) but adrenaline given intravenously in low doses produced both pressor and cardio-accelerator effects

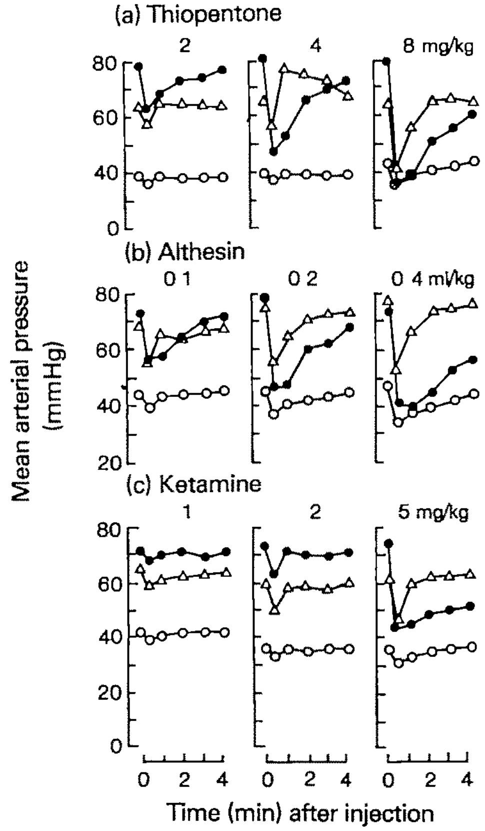

Figure 10 Comparison of the effects of (a) thiopentone (b) althesin and (c) ketamine on the mean arterial pressure of the pithed or decerebrate rabbit. (●) Decerebrate rabbits, (○) pithed rabbits, (△) same pithed rabbits but during continuous stimulation of the sympathetic outflow at T8 (5 Hz); $n=5$ throughout. Standard error bars have been omitted for clarity but s.e. mean in each case was similar to that indicated in Table 1.

(authors' unpublished observations) suggesting that the absence of the adrenal response in the preparation is due to failure to stimulate release of catecholamines from the adrenals rather than the absence of response to released catecholamines. However, the absence of the adrenal response is of advantage as it enables the production of a maintained sympathetic vasopressor response.

When the thoraco-lumbar sympathetic outflow of the pithed rat was stimulated continuously, the pressor response was not well maintained due to the effects of adrenal catecholamines on the heart (Gillespie & Muir, 1967a, b). With the pithed rabbit, stimulation at T8 produced no change in heart rate but a maintained rise in arterial pressure which was due solely to the direct vasopressor effect of the sympathetic nerves.

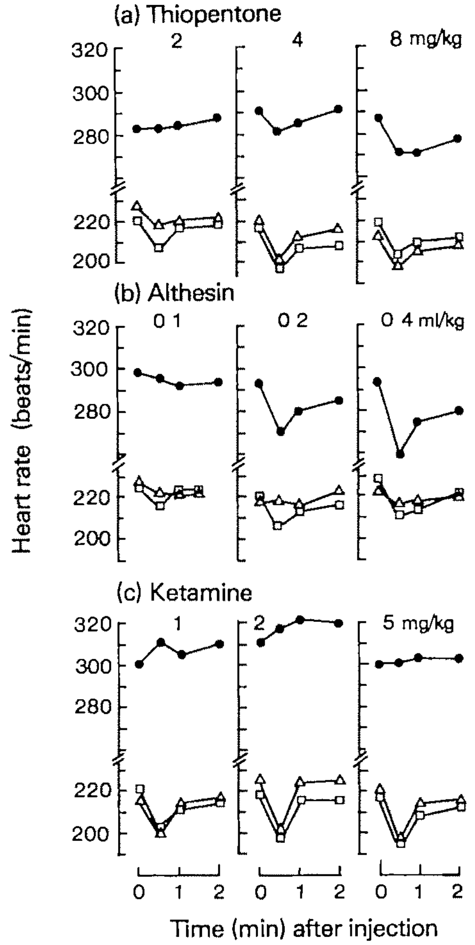

Figure 11 Comparison of the effects of (a) thiopentone, (b) althesin and (c) ketamine on the heart rate of the pithed or decerebrate rabbit. (●) Decerebrate rabbits, (□) pithed rabbits, (△) same pithed rabbits but during continuous stimulation of the sympathetic outflow at T8 (5 Hz); $n=5$ throughout. Standard error bars have been omitted for clarity but s.e. mean in each case was similar to that indicated in Table 1.

This further contrasts with the pithed rat where it was not possible to produce a marked elevation of blood pressure without cardiac stimulation (Gillespie *et al.*, 1970).

With continuous stimulation at T8 the resulting increase in peripheral resistance increased the afterload on the heart and thus increased the work of the heart as expected from Starling's Law and reflected by a maintained increase in left ventricular dP/dt_{max} (Linden & Snow, 1974). With continuous stimulation, the cardiovascular system reached a new equilibrium position within a few minutes, whereby the heart rate remained as before, the arterial pressure was elevated and the heart contracted more forcibly to maintain the cardiac output. At this point the peripheral resistance was comparable to that in the decerebrate rabbit but the heart rate and myocardial contractility were lower, presumably due to the effects of cardiac sympathetic tone in the decerebrate preparations. This resulted in a lower cardiac output in the pithed preparation although the stroke volume was in fact greater than in the decerebrate preparation (see Table 3).

A further contrast between the preparations which is relevant to the effects of drugs is that the regional distribution of the peripheral resistance will be different. In the pithed rabbit the only nerves contributing will be those emanating from the lower thoracic region and these will be active only at the stimulator frequency of 5 Hz, whereas in the decerebrate rabbit vasoconstriction will be more generalized and the frequency of nerve traffic variable. In this context it is worth noting that continuous stimulation of the thoracic outflows produced not only an increase in peripheral resistance but also an increase in cardiac output. A possible explanation for this is that such stimulation will not distinguish between sympathetic fibres to arterioles and those to veins. Whereas arteriolar constriction will increase peripheral resistance, an increase in venomotor tone will produce an increase in venous return and lead to an increase in cardiac output. At the stimulation frequency of 5 Hz used here, the relative effect on capacitance vessels compared with resistance vessels will be particularly marked since the former are relatively more sensitive to such low frequencies of stimulation (Mellander, 1960).

As with the pithed rat and cat (Gillespie *et al.*, 1970), the nature of the nerves stimulated by the pithing rod electrode could be verified by the use of specific blocking drugs. All responses to stimulation with the pithing rod electrode were abolished by hexamethonium, whereas the corresponding responses to the agonist drugs noradrenaline and isoprenaline were unaffected, verifying that stimulation was of the preganglionic vertebral outflows. The dose of hexamethonium required to abolish the responses from different organs varied considerably as was found previously in the pithed rat (Gillespie & McGrath, 1973). The vasopressor responses to stimulation at T8 and to noradrenaline were inhibited by the α-adrenoceptor antagonist phentolamine, whereas the cardio-accelerator responses to stimulation at T3 and to noradrenaline or isoprenaline were inhibited by the β-adrenoceptor antagonist propranolol.

The responses to stimulation of adrenergic nerves or to injected noradrenaline could be potentiated by cocaine. Cocaine has been shown to potentiate responses to noradrenaline in the pithed rat by blocking the uptake of noradrenaline into adrenergic nerve terminals (Muscholl, 1961; Simpson, 1975). Blockade of noradrenaline uptake is also likely to be the mechanism in the rabbit since both α- and β-adrenoceptor effects were potentiated and cocaine, at the dose used, had no effects on arterial pressure or heart rate on its own. It was interesting to note that during continuous stimulation of the vasopressor nerves at T8, cocaine did elevate the arterial pressure. This example illustrates the type of peripheral response which can be detected by this preparation but which might be absent in the intact animal due to the buffering effect of the baroreceptor reflexes, and indicates that either inhibition or potentiation of nerve responses can be demonstrated.

The results indicate that the intravenous anaesthetic agents tested have both central and peripheral actions, each of which, at anaesthetic doses, can affect the cardiovascular system.

In the pithed rabbit the effects on peripheral cardiovascular responses were dose-related. At low doses there was some evidence of potentiation of the responses to noradrenaline while at the higher doses depression of sympathetic nerve responses was found.

Evidence from *in vitro* preparations has demonstrated that ketamine can potentiate adrenergic responses by blockade of neuronal noradrenaline uptake (Nedergaard, 1973; Montel, Starke, Gorlitz & Schumann, 1973). However, with intravenous administration of ketamine to the pithed rat it was shown that the cardiovascular effects depended on a balance between the potentiating effect of noradrenaline uptake blockade and the depressant effects on synaptic transmission. Thus doses of ketamine which potentiated responses to injected noradrenaline depressed responses to sympathetic nerve stimulation at both the ganglion synapse and at the post-ganglionic nerve-muscle junction (Clanachan & McGrath, 1976).

Similar factors appear to have operated in the present study with sympathetic nerve responses being depressed at anaesthetic doses by ketamine, althesin and pentobarbitone.

From the resting pithed rabbit it was clear that an immediate peripheral cardiovascular depression followed injection of the anaesthetic agents tested. Heart rate and left ventricular dP/dt both fell on a time scale at least as fast as the fall in arterial pressure, indicating that a major part of the depression was exerted on the heart. A direct depression of the myocardium by several intravenous anaesthetics including ketamine and barbiturates is well documented (Dowdy & Kaya, 1968; Fischer, 1973; Schwartz & Horwitz, 1975). With the technique employed it was not possible to rule out completely some peripheral vasodilatation. The reason for this was that since the effects were so transient, cardiac output and hence peripheral resistance could only be accurately measured 1 min after injection by which time recovery of the arterial pressure was almost complete and no significant changes in peripheral resistance were detected.

With continuous sympathetic stimulation, any effects of the agents on the peripheral sympathetic pathway might be expected to appear. In fact no statistically significant difference stood out when compared with the effects in the resting pithed rabbit. The effects of vasopressor nerve stimulation might, however, have been expected to be potentiated by the lower doses of thiopentone and depressed by the highest doses of althesin and ketamine since these latter effects were found with short periods of vasopressor nerve stimulation.

This may indicate that the responses to nerves in continuous operation are relatively resistant to modification by drugs when compared with the responses to bursts of activity from inactive nerves and suggests caution when interpreting the effects of drugs in the latter situation.

When the effects of these agents were tested on decerebrate rabbits, the depressor effects were larger and longer lasting. This was expected from previous studies where sympathetic nerve activity was monitored in decerebrate or pentobarbitone anaesthetized rabbits and was seen to fall amost in parallel with the arterial pressure (McGrath *et al.*, 1975a; Mackenzie *et al.*, 1976). Taken together with these latter studies, the present results indicate that the depressor effect in the decerebrate rabbit of thiopentone, althesin and ketamine consists of 2 phases viz.

(1) An initial fall in arterial pressure due to the immediate post-injection depression of the heart and which is virtually over within 1 min, (2) a reduction in central sympathetic discharge which is a few seconds slower to reach its peak than (1), presumably due to the extra time required for the agent to reach the central nervous system, and which lasts for several minutes.

The corresponding effects on the heart rate are more difficult to interpret due to the interaction between the sympathetic and parasympathetic activity in the case of the decerebrate rabbits. However, the effects on the heart rate of pithed and decerebrate rabbits were similar, indicating no dramatic effects other than that of the initial myocardial depression. The one exception to this was with ketamine where the heart rate of the decerebrate preparations was not significantly affected but that of the pithed rabbits was. This may point to an interaction between the two branches of the autonomic nervous system following ketamine which could result from a selective inhibition of the parasympathetic component of the baroreceptor reflex (McGrath *et al.*, 1975a).

The similarity in the peripheral effects of althesin and ketamine is interesting in view of their known contrasting effects on the blood pressure of both man and several animal species (Domino *et al.*, 1965; McCarthy, Chen, Kaump & Ensor, 1965; Clarke *et al.*, 19 1). In the conscious rabbit, at the highest doses employed in the present study and at the same time after injection of the agent, ketamine increased (McGrath *et al.*, 1975a) and althesin decreased (Mackenzie *et al.*, 1976) arterial blood pressure. However, in the decerebrate rabbit both agents produced a parallel reduction in arterial blood pressure and sympathetic nerve activity (McGrath *et al.*, 1975b; Mackenzie *et al.*, 1976). Taken together these latter studies suggested that ketamine and althesin have similar effects on the medullary control of blood pressure, causing a reduction in the central sympathetic output, but that in the intact conscious animal some further effect of ketamine mediated via the central nervous system leads to a net pressor response.

In contrast to the other agents used, thiopentone has been shown to produce a direct constriction of arterial tissue (Burn & Hobbs, 1959; Price & Price, 1962; Altura & Altura, 1975). As this effect was potentiated by cocaine and abolished by an α-adrenoceptor antagonist or by reserpine-treatment (Burn & Hobbs, 1959), it has been suggested that thiopentone produces noradrenaline release. Since thiopentone does not elevate efferent sympathetic activity (Mackenzie *et al.*, 1976), it is likely that the increased peripheral resistance, which has been shown to result from administration of thiopentone (Etsten & Li, 1955; Fieldman, Ridley & Wood, 1955), arises from a peripheral effect. The present study confirms that thiopentone, at anaesthetic doses, can produce potentiation of sympathetic vasopressor responses which is of a purely peripheral origin. However, under the conditions employed here, there was no evidence for a sympathomimetic effect of thiopentone itself.

In conclusion a comparison of pithed and decerebrate preparations can be employed to locate the different sites at which relatively non-specific pharmacological agents such as anaesthetics may exert their effects. It must be borne in mind, however, that much more subtle and complex effects may manifest themselves in the conscious animal or patient (Price, 1960; McGrath *et al.*, 1975b).

Technical assistance was provided by Mr G.M. McCreaddie. This work was supported by the Medical Research Council and the Medical Research Funds of the University of Glasgow.

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(Received December 20, 1976.
Revised March 31, 1977)

Br. J. Pharmac. (1977), **61**, 213–215

CENTRAL AND PERIPHERAL NORADRENALINE IN THE TWO KIDNEY MODEL OF RENOVASCULAR HYPERTENSION IN THE RAT

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1 Plasma noradrenaline concentrations were similar in rats following unilateral renal arterial constriction (two kidney Goldblatt model) and in sham-operated control rats.

2 The development of hypertension was not affected by pretreatment with intracisternal injections of 6-hydroxydopamine.

3 These data suggest that sympathetic mechanisms do not contribute to the development of hypertension in this model.

Introduction

The importance of central noradrenergic neurones has been established in several models of experimental hypertension (Haeusler, Finch & Thoenen, 1972; Chalmers, Dollery, Lewis & Reid, 1974; Lewis, Dargie & Dollery, 1975). In the model of renovascular hypertension in the rat in which the renal arterial constriction is accompanied by contralateral nephrectomy (one kidney Goldblatt), pretreatment with intracisternal 6-hydroxydopamine (6-OHDA), which causes a long-lasting depletion of central noradrenergic neurones, has been shown to prevent the development of hypertension (Haeusler *et al.*, 1972; Dargie, Franklin & Reid, 1976). Pretreatment with intracisternal 6-OHDA has also prevented the increase in plasma noradrenaline (NA) concentrations which accompanies the increase in blood pressure (Dargie *et al.*, 1976).

In the model of renovascular hypertension in which renal arterial constriction only is performed, the other kidney being left intact (two kidney Goldblatt), the hypertension is primarily dependent on increased activity of the renin angiotensin system (Brunner, Gavras & Laragh, 1974).

In the present study we have assessed the contribution of the sympathetic nervous system to the hypertension in the two kidney model by measuring plasma NA concentrations at various times following renal arterial constriction and have examined the effect of pretreatment with intracisternal 6-OHDA both on the development of hypertension and on plasma NA concentrations.

Methods

Male Wistar rats weighing 200 g had a silver clip 0.009 inch wide placed over the left renal artery while control rats underwent a sham operative procedure involving placement of a broad non-constricting clip over the left renal artery. Blood pressure was measured by tail plethysmography using a programmed electrosphygmomanometer and pneumatic pulse transducer (Narco Biosystems Inc.). At 24 h 7, 24 and 28 days after operation, groups of 6 to 8 renal arterial clip rats together with their sham-operated controls were killed by decapitation 4 to 6 h after the blood pressure measurement (see Roizen, Weise, Moss & Kopin, 1975). The first 1 ml of 'arterialized' blood from the trunk was collected in ice-cold heparinized tubes for estimation of NA by the method of Henry, Starman, Johnson & Williams (1975).

Two groups of 20 rats were pretreated 14 days before renal arterial manipulation with either two intracisternal injections of 6-OHDA 200 μg or an identical volume (20 μl) of ascorbate saline vehicle the interval between injections being 48 hours. Blood pressure was measured by tail plethysmography at 1 and 7 days following renal arterial manipulation. Blood pressure and plasma NA concentrations were measured in two further groups of rats pretreated with either intracisternal 6-OHDA or vehicle at 7 days following renal arterial manipulation.

Results

Blood pressure and plasma noradrenaline concentration

In the first series of experiments mean systolic blood pressure was significantly elevated in the renal arterial clip group at 24 h, 7, 14 and 28 days reaching a maximum of 161 ± 5 mmHg at 7 days following renal

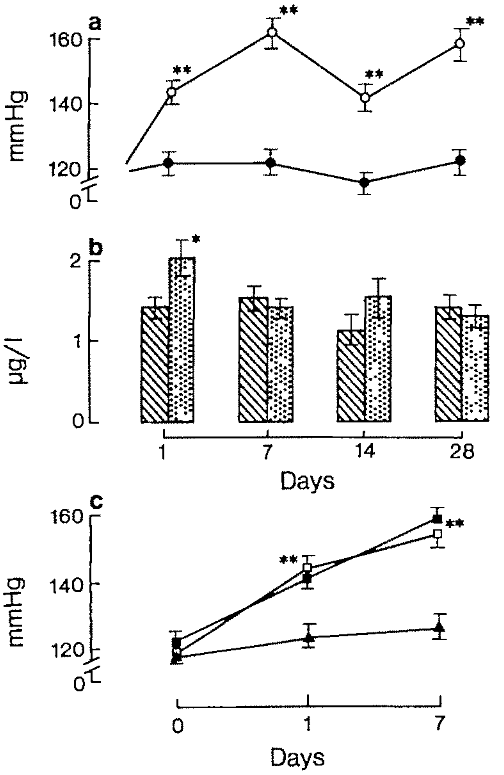

Figure 1 (a) Systolic blood pressure (mean) in renal arterial clip rats (○) and sham operated controls (●) at 1, 7, 14 and 28 days after renal manipulation. Vertical lines show s.e. mean. (b) Plasma noradrenaline concentrations (mean) in renal arterial clip rats (hatched columns) and sham-operated controls (stippled columns) at 1, 7, 14 and 28 days after renal manipulation. Vertical lines show s.e. mean. (c) Systolic blood pressures (mean) in renal arterial clip rats pretreated with intracisternal 6-hydroxydopamine (■) or vehicle (□) at 1 and 7 days after renal arterial manipulation and control unoperated rats (▲). Vertical lines show s.e. mean. $^*P<0.05$; $^{**}P<0.001$ (Student's t test).

arterial manipulation ($P<0.01$). The sham-operated group did not become hypertensive at any time during the experiment (Figure 1a). At 7 days when the blood pressure was highest, plasma NA in the renal arterial clip group was 1.50 ± 0.20 μg/l which was not significantly different from the value of 1.52 ± 0.18 μg/l obtained in the sham-operated control group at the same time. Although at 24 h after operation plasma NA was slightly but significantly higher in the sham-operated group than in the renal arterial clip group, at no time was the plasma NA level significantly greater in the renal clip group than in the sham-operated controls (Figure 1b).

Effect of intracisternal 6-hydroxydopamine

Mean systolic blood pressure in the first group of renal arterial clip rats pretreated with intracisternal 6-OHDA was not significantly different from renal arterial clip rats pretreated with intracisternal vehicle at either 24 h or 7 days following operation, values being 144 ± 5.0 mmHg and 146 ± 6 mmHg respectively at 24 h and 159 ± 5 mmHg and 150 ± 5 mmHg respectively at 7 days. All these values were significantly greater than in sham-operated rats whose blood pressures were 119 ± 4 mmHg and 129 ± 4 mmHg at 24 h and 7 days respectively following operation ($P<0.001$) (Figure 1c). Mean systolic blood pressure in the further group of renal arterial clip rats pretreated with intracisternal 6-OHDA was not significantly different from renal arterial clip rats pretreated with intracisternal vehicle, values being 129 ± 4 mmHg and 136 ± 3 mmHg respectively. Both these values were significantly higher than their sham-operated controls whose pressures were 113 ± 8 mmHg and 116 ± 3 mmHg ($P<0.001$). Plasma NA concentrations in both renal arterial clip groups were not significantly different from sham-operated animals pretreated with intracisternal vehicle, values being 1.58 ± 0.44, 1.30 ± 0.22 and 1.55 ± 0.34 μg/l respectively. The only difference was that plasma NA in the sham-operated rats pretreated with intracisternal 6-OHDA was lower than the other three operated groups at 0.81 ± 0.25 μg/l ($P<0.05$). However, this value was not significantly different from that of a non-operated normotensive control group killed at the same time in which plasma NA was 0.97 ± 0.25 μg/litre.

Discussion

In this study we have shown that the development of hypertension after renal arterial constriction with an intact contralateral kidney (two kidney Goldblatt) is not associated with an increase in plasma NA levels, nor is the hypertension modified by pretreatment with intracisternal 6-OHDA which depletes central noradrenergic neurones. There have been few studies of sympathetic function in the two kidney Goldblatt model but in one recent study neonatal sympathectomy had no effect on the development of hypertension in this model (Provoost, Bohus & de Jong, 1976) confirming the lack of dependence of the raised blood pressure on sympathetic mechanisms. These data contrast sharply with previous experience with the one kidney Goldblatt model in the rat in which an increase in plasma NA accompanies the development of hypertension and in which pretreatment with intracisternal 6-OHDA prevents not only the development of hypertension but also the increase in plasma NA concentrations (Dargie *et al.*, 1976). Similar data have been obtained in the deoxycorticosterone acetate (DOCA) salt model in the rat (Reid, Zivin & Kopin, 1975) and increased plasma NA concentrations have also been described in spontaneous hypertension in the rat (Grobecker, Roizen, Weise, Saavedra & Kopin, 1976). Pretreatment with intracisternal 6-OHDA prevents hypertension not only in the DOCA salt and the one kidney Goldblatt models but also in the perinephritis model in the rabbit in which bilateral cellophane wrapping of the kidneys results in interstitial nephritis. This suggests that in these models the development of hypertension results from a centrally mediated increase in sympathetic activity.

In our previous studies with the one kidney Goldblatt model, plasma NA was high in both the renal arterial clip and sham-operated groups at 24 h after the operative procedure (Dargie *et al.*, 1976). This was carried out under ether anaesthesia and we attributed the elevation of plasma NA at this stage to anaesthesia and operative stress, and the high plasma NA in the sham-operated group in the present study at 24 h could be related to similar mechanisms. The lower level in the renal arterial clip group cannot be explained at present and further studies will be required to determine its physiological significance, if any.

In the two kidney Goldblatt model, dependence on the renin angiotensin system has been suggested by increased plasma levels of renin and angiotensin II (Koletsky, Paulico & Rivera-Velez, 1971) and more recently confirmed by the use of specific angiotensin II antagonists (Brunner *et al.*, 1974). Angiotensin II can stimulate sympathetic activity centrally via an action on the area postrema in the mid brain (Joy & Lowe, 1969) and it is possible that any resulting increase in sympathetic activity might contribute to the development and maintenance of hypertension in the two kidney model. However, the absence of any increase of plasma NA concentration in the renal arterial clip animals in the present study and the lack of effect on the development of hypertension of depletion of central noradrenergic neurones by intracisternal 6-OHDA indicate that the central and peripheral sympathetic nervous system do not play a major role in the development of hypertension in the two kidney Goldblatt model of experimental hypertension in the rat.

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(Received January 19, 1977.
Revised April 30, 1977)

Br. J. Pharmac. (1977), **61**, 217–222

SPASMOGENIC AND POTENTIATING ACTIONS OF SOME AMINO ACIDS ON THE GUINEA-PIG MYOMETRIUM

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1 Thirty-three amino acids were applied separately in concentrations of 2 to 10 mM to guinea-pig uterine horns *in vitro* at pH 7.4. About half the acids regularly produced contractions.

2 Glycine and the straight-chain L-α-amino acids up to norleucine were active (longer ones not tested); D-isomers were less potent or inactive in these concentrations. The ω-amino acids γ-aminobutyric acid (GABA) and δ-aminovaleric, and the α,ω-diamino acids L-α, β-diaminopropionic and L-α,γ-diaminobutyric were active, whereas others of similar chain-length such as β-alanine and lysine were not. The diacidic acids, glutamic and homocysteic, were more active than the amido-amino acids, glutamine and asparagine. Histidine and phenylalanine showed little or no activity.

3 The use of appropriate blocking agents indicated that the responses to representative acids were not mediated by histamine, 5-hydroxytryptamine, acetylcholine, noradrenaline or by prostaglandins. Attempts to block the actions of glycine and GABA with strychnine, thebaine, picrotoxin, bicuculline or tetramethylenedisulphotetramine (TETS) were unsuccessful.

4 When some of the acids that were spasmogenic at 2 to 10 mM were applied at sub-spasmogenic doses, they transiently potentiated other spasmogens such as oxytocin or acetylcholine. This effect was also shown by a mixture of amino acids at approximately the normal plasma concentrations.

5 There is some similarity between the spasmogenic activities of different amino acids and their known abilities to depolarize neurones.

Introduction

Amino acids may stimulate smooth muscle (Jones, 1962; Klingenberg, 1966; Lewis, McMartin, Rosenthal & Yates, 1972). Among these, the only extensive study of amino acids in general was that of Lewis *et al.* (1972), who were concerned to identify the non-histamine active substances in the stratum corneum of human skin, and to define the receptors on which the substances acted; these authors used the guinea-pig ileum as their test object. We, however, wished to study a possible parallelism between the actions of amino acids in stimulating smooth muscle, and in depolarizing cells in the central nervous system (CNS). Consequently, we have compared several acids known to be active on CNS cells with others not known to be active. Preliminary trials suggested that the guinea-pig uterus was a suitable test object. Some of the results have been demonstrated to the British Pharmacological Society (Ishizawa & Pickles, 1975) or communicated to the Physiological Society (Bedwani, Pickles & Suwankrughasn, 1976).

Methods

Uterine horns from non-oestrous guinea-pigs weighing 420–650 g were suspended in a modified Krebs-Henseleit solution at pH 7.4, maintained at 36–37°C and normally containing Ca^{2+} and Mg^{2+} each 1 mM, the $[Mg^{2+}]$ being increased if necessary to give a conveniently low degree of spontaneous activity in the preparations. Contractions were recorded either isometrically or by means of a lightly spring-loaded lever on a kymograph. The amino acids or their sodium salts or hydrochlorides (Sigma Chemical Co.) were freshly made up as isotonic solutions in deionized water and adjusted if necessary to pH 7.4. Volumes of these solutions from 1/50 to 1/10 of the organ-bath volume were added directly to give the higher concentrations of amino acids; for the lower concentrations, the original amino acid solutions were diluted ten-fold with isotonic NaCl solution. Control

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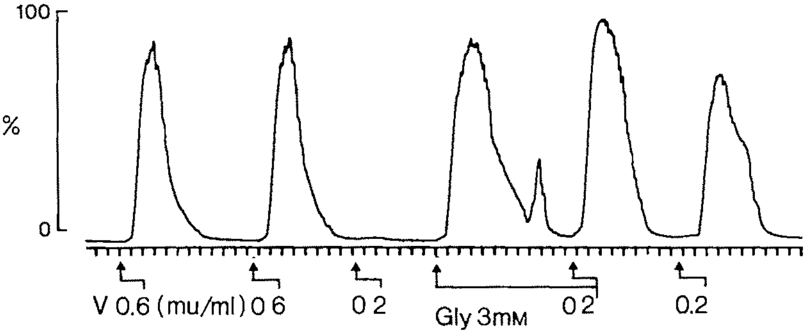

Figure 1 Guinea-pig uterine horn *in vitro*: effects of glycine (3 mM bath concentration) and vasopressin (V, doses as indicated) alone and in the presence of glycine. Time-marker 1 minute. On vertical scale, 100% =47 mm.

applications of NaCl or isotonic sucrose solution had no effect. Tests with vasopressin or oxytocin were always included as a check of the excitability of the preparations. Each acid was tested in at least three separate experiments but some, especially glycine and γ-aminobutyric acid (GABA), in many more.

Results

Potency groups

A typical response to glycine, one of the more active of the amino acids tested, is shown in Figure 1. The responses of the guinea-pig myometrium in general are less precisely dose-dependent than those of the ileum, and smooth 'dose-response' curves may require a large number of trials. For our present purposes, we have simply grouped the acids broadly in terms of their relative potencies. Thus glycine fell into the most potent group, in which concentrations within the range 2 to 5 mM usually caused strong but not necessarily maximal contractions. In the less potent group, 5 to 10 mM was needed for a similar response; while for the least potent acids, 10 to 15 mM gave little or no response.

The potencies thus defined indicate the responses in most but not in all experiments. In a few experiments, an acid might give responses either greater or less than would be suggested by the potency-group to which it was assigned. Where this variability was particularly noticeable, it is indicated in Table 1 by a range of potency groups. Another cause of variability was the differences between uteri in their spontaneous contractile activity: preparations showing greater activity were more sensitive to amino acids but gave

Table 1 Concentrations of acids needed to produce contractions of the guinea-pig myometrium, as described in the text

α-Amino acids	*ω-Amino acids*	*α,ω-Diamino acids*
Glycine + +		
L-α-Alanine + +	β-Alanine 0	L-α,β-Diaminopropionic +
L-α-Aminobutyric + +	GABA 0 – + +	L-α,γ-Diaminobutyric + +
L-α-Aminovaleric (norvaline) +	δ-Aminovaleric + +	L-α, δ-Diaminovaleric (ornithine) 0
L-α-Aminocaproic (norleucine) + +	ε-Aminocaproic 0	L-α, ε-Diaminocaproic (lysine) 0
Branched-chain amino acids	*Diacidic amino acids*	*Amido amino acids*
α-Amino*iso*butyric +	L-Glutamic + –+ +	L-Glutamine 0
L-Valine 0	L-Aspartic 0	L-Asparagine 0
L-Leucine 0 – +	DL-Homocysteic +	
*Others, including non-amino acids**	*N*-acetyl-L-aspartic 0 – +	
β-Aminobutyric 0	Proline +	Serine +
β-Hydroxybutyric* 0	Histidine 0 – +	Kainic + +
γ-Hydroxybutyric* 0 – +	Phenylalanine 0	δ-Aminolaevulinic + +

Key: + + 2 to 5 mM; + 5 to 10 mM; 0 > 10 mM, or no response.

recordings that were more difficult to assess quantitatively. This was particularly well illustrated by a few experiments in which the two horns of the same uterus, treated identically so far as was known, nevertheless showed different degrees of spontaneous activity.

Potency in relation to structure

Table 1 shows the acids we tested, classified according to their chemical nature and with their potencies indicated in terms of these three groups.

All the neutral straight-chain L-α-amino acids we tested were active. Of the corresponding ω-amino acids from β-alanine to ε-aminocaproic acid, only δ-aminovaleric was regularly active, though GABA was equally potent in some experiments. The first two L-α,ω-diaminoacids, namely L-α,β-diaminopropionic and L-α,γ-diaminobutyric were active as might be expected; but surprisingly neither L-α,δ-diaminovaleric (ornithine) nor L-α,ε-diaminocaproic (lysine) was active in our normal experimental conditions. Three D-α-amino acids were tested, namely D-α-alanine, D-α-aminobutyric acid and D-norleucine, and all proved to be less active than their L-enantiomers; D-lysine was also inactive. The branched-chain acids α-amino*iso*butyric, L-valine and L-leucine were either inactive or less potent than the corresponding straight-chain acids.

Among diacidic substances, L-glutamic and DL-homocysteic were active; the activity of glutamic acid contrasted with the inactivity of glutamine. Aspartic acid, its amide asparagine, and *N*-acetyl-L-aspartic acid, were less potent than glutamic acid.

Table 1 also shows the results with a number of other acids, including proline (an imino acid) and others that are not amino acids. In comparisons on the same uteri, GABA was shown to be more potent than γ-hydroxybutyric, β-aminobutyric and β-hydroxybutyric acids. Kainic acid which is a powerful convulsant (Shinozaki & Konishi, 1970) seemed to be more potent than glutamic acid to which it is structurally related, but the amount available did not allow full testing.

Action not mediated by other spasmogens

In order to eliminate the possibility that the responses were mediated by the liberation of other spasmogens, representative amino acids were tested in the presence of the specific antagonists mepyramine (2 µM), hexamethonium (10 µM), atropine (1 µM), phentolamine (1.6 µM), and methysergide (0.4 µM). The myometria were then unresponsive to histamine, acetylcholine, noradrenaline and 5-hydroxytryptamine, but they still responded normally to glycine, α,γ-diaminobutyric acid, and δ-aminolaevulinic acid. Other experiments in which the antagonists were applied individually showed that responses to GABA were unaffected. Histamine responses were blocked by mepyramine but not by burimamide (20 µM), showing that they were mediated by H_1-receptors only, and in these circumstances it was not surprising that burimamide did not alter the responses to glycine. In two experiments, indomethacin (6 and 14 µM) was present throughout in the Krebs-Henseleit solution for one uterine horn while the other was used as a control; there was no difference in the direct responses to glycine, GABA or glutamate, suggesting that these responses were not mediated by endogenous prostaglandin production. The prostaglandin antagonist 7-oxa-prost-13-ynoic acid was spasmogenic on the myometrium, and another antagonist diphloretin phosphate (DPP) proved to be not highly specific on this tissue; consequently, clear evidence could not be obtained for or against the possible participation of previously formed prostaglandins in the amino acid responses.

Because some effects of glycine or GABA in the CNS may be blocked by strychnine, thebaine, picrotoxin, bicuculline or tetramethylenedisulphotetramine (TETS), these substances were used in attempts to block the action of glycine and GABA on the myometrium. The results were difficult to interpret, because 1 mM or even lower concentrations of these substances either caused non-specific depression or were themselves spasmogenic. However, no trace of specific antagonism was ever seen.

Potentiation of other spasmogens

Some amino acids were found to potentiate other spasmogens. An example is seen in Figure 1, where a previously sub-threshold dose of vasopressin, 0.2 mu/ml, gave a large response when added in the presence of glycine, and a smaller but definite one again 9 min after the glycine had been washed out of the organ bath. In neither instance can the effect be readily explained by simple summation of responses. Further examples are shown in Figure 2, where the direct responses to GABA and to glycine were small but the responses to vasopressin were markedly potentiated. Figure 3 shows the results of another series of experiments, in which GABA concentrations less than one-tenth of the threshold for direct responses potentiated the spasmogenic effect of added Ca^{2+}. Similar results have been obtained with glycine.

The phenomenon seen in Figure 1, in which the potentiation apparently out-lasted the presence of the amino acid in the organ bath, varied greatly from experiment to experiment and possibly from one amino acid to another. In a few instances, it was as marked and as prolonged as the 'enhancement' following the application of prostaglandin E_1 or E_2 to the guinea-pig myometrium (Clegg, Hall & Pickles, 1966); whereas in other experiments it was absent or led rapidly into a period of decreased responses. This variability was not studied in detail.

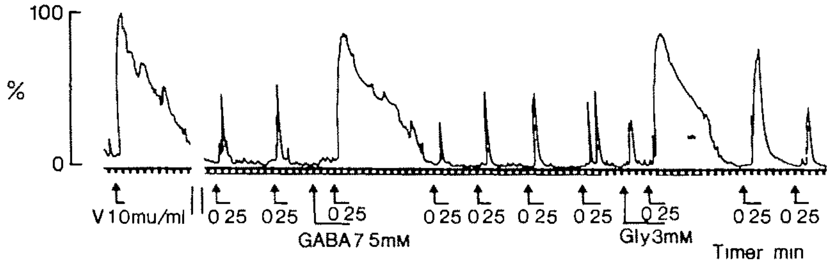

Figure 2 Guinea-pig uterine horn *in vitro*: responses to vasopressin (V, doses as indicated), and the effects of γ-aminobutyric acid (GABA) 7.5 mM and glycine (Gly) 3 mM on those responses. Gap between sections, 20 minutes. On vertical scale, 100% = 33 mm.

For a final short series of experiments two amino acid concentrates were prepared, one 'active' and the other 'inactive', such that on appropriate dilution the amino acid concentrations would approximate to those found free in the plasma of various species (Altman & Dittmer, 1974). The 'active' mixture comprised some of the acids given the + or + + rating in Table 1; on dilution it contained alanine 0.3, glycine 0.2, proline 0.2, serine 0.1, glutamic acid 0.05, norleucine 0.05 and α-aminobutyric acid 0.02 mM. Other acids rated + or + + were not included because their concentrations in plasma are much lower, or zero. The 'inactive' mixture on dilution gave lysine 0.15, leucine 0.1, histidine 0.05, ornithine 0.05, phenylalanine 0.05, and valine 0.02 mM. The results shown in Figure 4 were typical: the addition of the mixture of 'active' amino acids in plasma concentrations simultaneously with the oxytocin or acetylcholine increased the responses to the latter, but a similar addition of 'inactive' acids had no apparent effect. The 'inactive' mixture did not diminish the effect of the 'active' mixture.

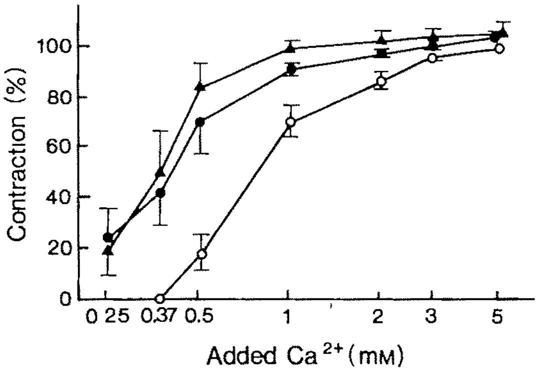

Figure 3 Guinea-pig uterine horns *in vitro* in a medium containing Ca^{2+} 0.1 mM and Mg^{2+} 3 mM, contracting in response to added $CaCl_2$ in the concentrations shown on the abscissa scale. (○) In absence of γ-aminobutyric acid (GABA), $n = 11$; (●) in presence of GABA 0.05 mM, $n = 5$; (▲) in presence of GABA 0.15 mM, $n = 5$. The contractions are expressed as percentages of the responses to the addition of 5 mM $CaCl_2$ in the absence of GABA. Vertical bars indicate ± s.e. mean.

More than half the amino acid content of the 'active' mixture consisted of glycine and alanine, both of which are among the most potent spasmogens. It might therefore be asked whether the potentiating effect of the 'active' mixture was largely or entirely due to either or both of these acids alone. However, an 'active' mixture from which the glycine and alanine had been omitted still gave definite potentiation, though less than that given by the complete mixture.

Effects on spontaneous myometrial activity

When a myometrial preparation is contracting and relaxing spontaneously, it may be difficult or impossible to distinguish between a superimposed spasmogenesis as such, and an induced increase in the amount of spontaneous activity. In either case, the effect is difficult to quantitate. We have not systematically studied such effects in the experiments described here, but (as mentioned before) incidental observations have suggested that amino acids may be apparently more potent when assessed on a spontaneously active preparation than on a quiescent one. Figure 5 shows this particularly clearly, since the 'active' mixture alone normally had no apparent effect on quiescent preparations; and it also accords with the conclusions in the paragraph above.

Discussion

Our experiments on the guinea-pig uterus broadly confirm those of Lewis *et al.* (1972) on the ileum, in that a wide range of amino acids, but not all, can strongly stimulate smooth muscle when added in concentrations from 2 to 10 mM. Although it is difficult to make exact comparisons between the two investigations, there may be some significant differences

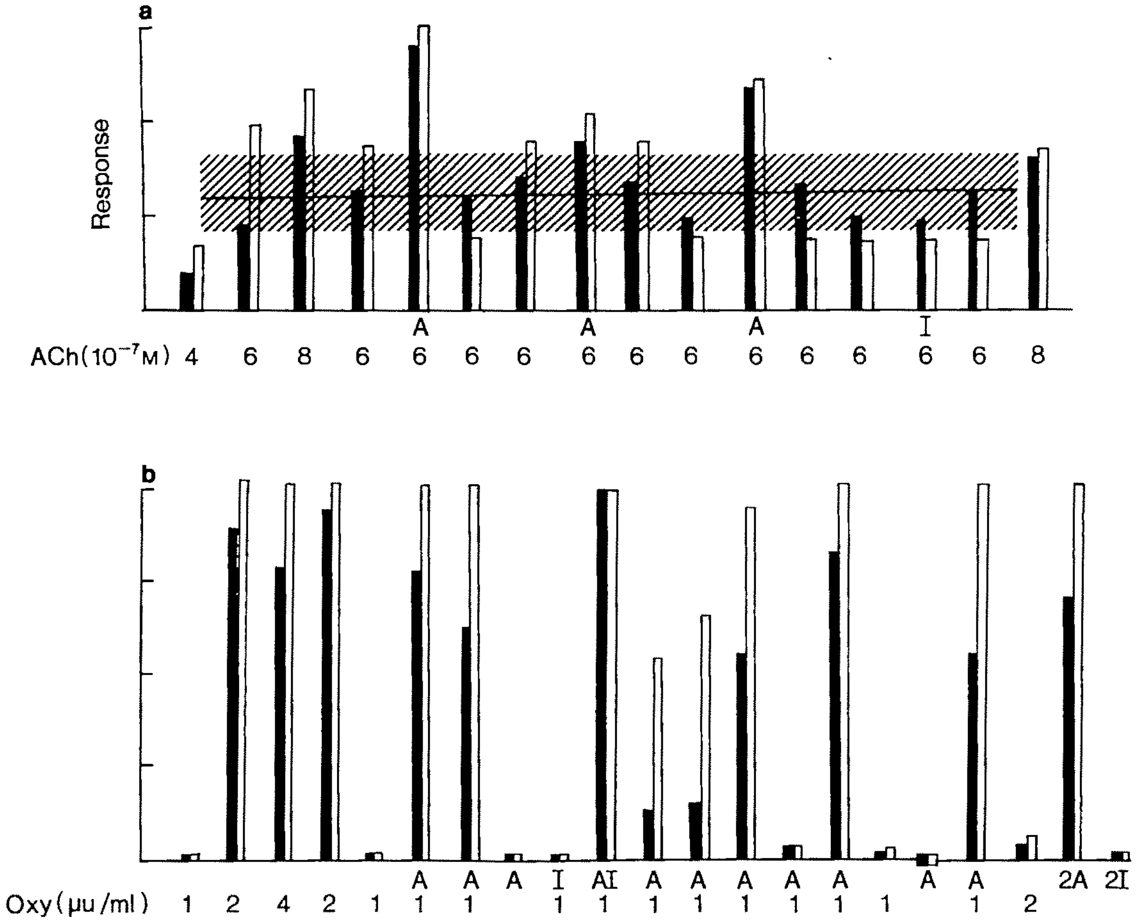

Figure 4 (a) Responses to acetylcholine (ACh) in the doses stated, with the simultaneous addition of the 'active' amino acid mixture (see text) at A, or the 'inactive' mixture at I. Each response is plotted both as the height on the recording (open column) and as the area under the curve from the time of application to 4 min after wash-out, when relaxation was nearly complete (solid column). The line, which is not quite horizontal, shows the calculated regression, on time, of the responses to the 6 x 10^{-7} M doses of acetylcholine alone (solid columns); the hatched area indicates ± s.d. (b) Responses of another preparation to oxytocin, expressed as in the upper graph. AI = simultaneous application of 'active' and 'inactive' mixtures; 2A, 2I = application of 'active' or 'inactive' mixture in double the previous concentration. The change from a very small to a near-maximal response with only a doubling of the dose, and the occasional apparently anomalous response such as the 14th one in (b), are characteristic of many guinea-pig uterine preparations *in vitro*. An apparent inhibitory phase, such as that following the application of A+I+oxytocin 1 (10th response), was seen in some other experiments but was not systematically investigated.

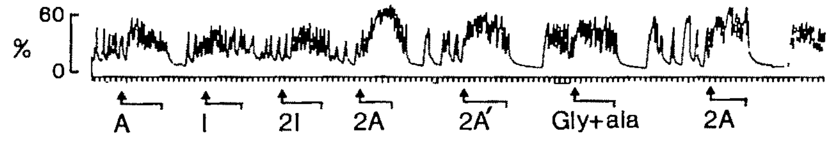

Figure 5 Effects on a spontaneously active preparation. A, I: 'active' and 'inactive' amino acid mixtures as before; A': as 'active' mixture but without glycine and alanine; Gly+ala: glycine 0.4 mM, alanine 0.6 mM, which when added to 2A' would make it the same as 2A. On vertical scale 60% = 12.6 mm.

in the results. In our experiments alanine was about twice as potent as glycine, whereas in theirs it was about seven times as potent; in our experiments glutamic acid was more potent than glutamine, whereas in theirs the reverse was true. The branched-chain acids, valine and leucine, which were of medium potency in their experiments were relatively inactive in ours. We tested homocysteic and kainic acids (which Lewis *et al.* (1972) did not test) because they are known to be depolarizers of central-nervous cells, and we found them active.

The use of specific blocking agents has shown that representative amino acids do not act by release of acetylcholine, noradrenaline, histamine or 5-hydroxytryptamine. Prostaglandin release is not a major factor though we cannot entirely exclude a minor effect of this kind. Lewis *et al.* (1972) postulated a receptor 'particularly sensitive to L-α-amino acids with a short side chain', GABA acting by a different mechanism. Our results are not incompatible with there being similar receptors in the myometrium; nevertheless, we are not positively proposing this. The range of active amino acids is wider and the effective concentrations much higher than one usually associates with specific action at receptors; for example, at post-synaptic glutamate receptors in the CNS. Other possible mechanisms may have to be considered. One might be of the type postulated by Klingenberg (1966), who suggested that cyclical fluctuations in a mechanism involving glutamate oxidation, Ca^{2+} storage and glutamine synthesis might explain the myometrial responses to exogenous glutamate. Alternatively the contractions might follow depolarization accompanying active uptake of amino acids, but we do not know of any studies of uptake in the myometrium with which to compare our results.

Such considerations are relevant to the comparison with the effects of amino acids on central neurones. For example, glutamate increases the activity of both types of cell, but with a great difference in effective dose. Glycine is best known as causing strychnine-sensitive hyperpolarization and inhibition in spinal cord neurones; but it may also cause strychnine-resistant depolarization probably leading to excitation (Nicoll, Padjen & Barker, 1976; Evans, Francis & Watkins, 1976), which corresponds more closely with its myometrial effect. The myometrium-stimulating potencies of various amino acids show a better correlation with their rates of metabolism by brain slices than with their uptake (Sadasivudu & Lajtha, 1970). We suggest that if the effects of amino acids on the myometrium correspond with any effect in the CNS, this is not with transmitter actions but with less specific effects which nevertheless are not given equally by all amino acids.

The concentrations of amino acids needed to produce definite contractions of the myometrium in the absence of other spasmogens are of the same order as those found free in whole-tissue extracts but are ten or more times as great as those in plasma. However, a mixture of acids in approximately the same concentrations as in plasma regularly potentiated other spasmogens. We propose that it is the excitability-modulating effect of some amino acids, rather than their direct spasmogenic action, that may represent a possible physiological effect on the myometrium.

We wish to thank Mr B. Shears and Mr P. Blanning for their skilled technical assistance. Gifts of 7-oxa-prost-13-ynoic acid from Dr J. Fried, DPP from Dr H. Fex, TETS from Dr J. F. Collins and kainic acid from Dr J. C. Watkins are also gratefully acknowledged.

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(Received January 21, 1977.
Revised April 1, 1977)

Br. J. Pharmac. (1977), **61**, 223–228

EVALUATION OF THE UPTAKE OF VARIOUS AMINES INTO STORAGE VESICLES OF INTACT HUMAN PLATELETS

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1 The uptake of various ^{3}H-labelled amines into a thrombin-releasable compartment of human platelets, thought to represent the platelet vesicular storage pool, has been evaluated. Measurable amounts of 5-hydroxytryptamine (5-HT), dopamine, tyramine, octopamine, and tryptamine accumulate in this pool, and also in a non-thrombin-releasable (cytoplasmic) pool during a 30 min incubation period with 10^{-5} M extracellular amine concentrations.

2 No differences in the accumulation of vesicular or cytoplasmic 5-HT, dopamine, and tyramine are found in platelets treated with deprenyl to inhibit platelet monoamine oxidase as compared to controls.

3 Extracellular tyramine or dopamine in concentrations as high as 10^{-5} M does not alter the initial rate of 5-HT uptake across the platelet plasma membrane. Similarly, sizable cytoplasmic pools of either amine do not alter the initial rate at which small amounts of 5-HT enter platelet cytoplasm or storage vesicles. 5-HT thus appears to be the preferred substrate for uptake into platelets and for movement from cytoplasm to vesicles.

Introduction

Human platelets appear to accumulate a variety of biogenic amines against a concentration gradient (for reviews see Murphy & Kopin, 1972; Sneddon, 1973). The reviews indicate the extent to which debate continues over whether amines other than 5-hydroxytryptamine (5-HT) can enter human platelet storage vesicles, although dopamine appears to enter vesicles in cat platelets (Boullin, McMahon & O'Brien, 1970), and isolated platelet vesicles of rabbit have been reported to accumulate dopamine and several other amines (Da Prada & Pletscher, 1969).

Recently-developed techniques, using formaldehyde fixation (Costa & Murphy, 1975) in conjunction with thrombin (Costa, Murphy & Kafka, 1977a), permit evaluation of the accumulation of radioactively-labelled substances into a platelet thrombin-releasable compartment, believed to represent the vesicular storage site. Using these methods, we have compared the distribution in intact platelets of 5-HT and other amines of biological interest in platelet cytoplasm and storage vesicles. The results of these studies suggested other experiments, also presented here, which examine the interaction between 5-HT and other amines at the platelet plasma and vesicular membranes.

Methods

Human platelets from normal volunteers were collected in citrate/disodium edetate (EDTA), and platelet-rich-plasma (PRP) was prepared by differential centrifugation at 4°C as described previously (Costa *et al.*, 1977a). All platelet counts were performed with an Electrozone Celloscope (Particle Data, Inc., Elmhurst, Ill.).

Calculation of the amounts of radioactively-labelled material in the vesicular compartment was made as described previously, using formaldehyde fixation and thrombin (Costa *et al.*, 1977a). Briefly, PRP was incubated with 2×10^{-7} M [^{14}C]-5-HT for 30 min at 37°C. Platelets were cooled to 0°C, spun into a pellet, and resuspended to a final concentration of 1–2×10^8/ml in a Tris-citrate buffer (Costa *et al.*, 1977a). Under these conditions, the [^{14}C]-5-HT can be used to follow the behaviour of the endogenous 5-HT stores, since in control preparations, the percentage release of label induced by thrombin serves as an index of the percentage release of the total number of vesicular storage sites (Costa & Murphy, 1976; Costa *et al.*, 1977a). The actual amount of vesicular amine was calculated as described previously (Costa *et al.*, 1977a).

Evaluation of amine uptake and 5-hydroxytryptamine loss

Platelets pre-labelled in PRP with 2×10^{-7} M [^{14}C]-5-HT as above were resuspended in buffer. ^{3}H-labelled amines (5-HT, 0.50 Ci/mmol; dopamine, 0.50 Ci/mmol; tyramine, 1.1 Ci/mmol; octopamine, 3.7 Ci/mmol; and tryptamine, 0.87 Ci/mmol) were added with the use of plastic plumpers (Costa *et al.*, 1977a) to 500 µl aliquots of resuspended platelets (held at 37°C) to a final concentration of 10^{-5} M for each of the amines tested. Thirty minutes after the addition of amine, aliquots were either fixed with formaldehyde, or treated with human thrombin (2 units/ml for 60 s) and subsequently fixed. ^{14}C- and ^{3}H-labelled material in the pellets was measured by conventional methods.

Evaluation of amine uptake in control and deprenyl-treated platelets

Platelets in PRP were prelabelled with 10^{-5} M cold 5-HT (30 min at 37°C). During the final 10 min of incubation, the irreversible monoamine oxidase (MAO) inhibitor, deprenyl (final concentration 10^{-4}M), was included in the incubation medium in some aliquots. Unpublished experiments in this laboratory have shown that this procedure results in essentially complete inhibition of the production of neutral and negatively-charged metabolites from tyramine and tryptamine by either intact, resuspended platelets or by platelet sonicates. Control and deprenyl-treated platelets were pelleted and resuspended in buffer. ^{3}H-amines (5-HT, dopamine, or tyramine; final concentrations 10^{-5} M) were added to 500 µl aliquots of resuspended platelets held at 37°C. Ten minutes after the addition of amine, platelets were either fixed or treated with thrombin and then fixed. The amount of ^{3}H-amine in platelets and platelet vesicles was evaluated as above.

Effect of extracellular dopamine or tyramine on uptake of extracellular 5-hydroxytryptamine into platelets

Uptake of 10^{-7} M [^{3}H]-5-HT into unlabelled platelets, resuspended in buffer, was measured over a 10 s period at 37°C (Costa & Murphy, 1975). Acid (0.01N HCl) was added to control aliquots immediately before [^{3}H]-5-HT addition; to other aliquots, unlabelled dopamine or tyramine in 0.01 N HCl (final concentrations either 10^{-6} M or 10^{-5} M) was added immediately before the addition of [^{3}H]-5-HT.

Effect of intra-platelet dopamine or tyramine on the uptake of exogenous 5-hydroxytryptamine

Platelets from PRP were resuspended in buffer without prelabelling with [^{14}C]-5-HT. In order to obtain appreciable cytoplasmic concentrations of [^{14}C]-dopamine or [^{14}C]-tyramine, aliquots were incubated for either 10 min or 30 min at 37°C with either [^{14}C]-dopamine (10^{-5} M) or [^{14}C]-tyramine (10^{-5}M) and cooled to 0°C. Pelleted platelets were resuspended to a final concentration of $1–2 \times 10^8$/ml in fresh, amine-free buffer. The uptake of 10^{-8} M exogenous [^{3}H]-5-HT was examined over a 1 min period in 500 µl aliquots at 37°C, using formaldehyde and thrombin as described above. Control platelets were similarly incubated for 30 min with diluent (0.01 N HCl), and resuspended in fresh buffer before the measurement of [^{3}H]-5-HT uptake. The percentage release of vesicular [^{14}C]-5-HT was evaluated in separate, PRP-prelabelled platelets from the same donor.

Effect of cytoplasmic 5-hydroxytryptamine on the uptake of exogenous 5-hydroxytryptamine

For this experiment, we wanted to compare the effect of cytoplasmic 5-HT with that of dopamine or tyramine on the movement of new 5-HT into vesicles. However, we could not employ the same protocol as used above, since relatively large pools of cytoplasmic 5-HT will migrate completely into vesicles following resuspension in an amine-free medium (Costa *et al.*, 1977a; Costa, Silber & Murphy, 1977b). In order to obtain widely differing cytoplasmic 5-HT concentrations, two different incubation protocols were employed. For incubation A, platelets in PRP were kept at 37°C for 20 min, pelleted, and resuspended. Resuspended platelets were incubated with 10^{-5} M [^{14}C]-5-HT for 30 min (37°C), and resuspended again in fresh buffer. For Incubation B, platelets in PRP were incubated with 10^{-5} M cold 5-HT for 20 min at 37°C and resuspended in fresh buffer. Resuspended platelets were subsequently incubated with 10^{-5} M [^{14}C]-5-HT (37°C for 30 min), and again resuspended in amine-free buffer. Control platelets were incubated following similar protocols, but diluent was used instead of 5-HT. The 1 min uptake of 10^{-8} M [^{3}H]-5-HT in the resuspended platelets from each incubation was measured at 37°C, as described above, and the amounts of both ^{3}H- and ^{14}C-labelled material in vesicular and cytoplasmic compartment were calculated.

Results

Table 1 delineates the cytoplasmic and vesicular concentrations of various ^{3}H-amines, and summarizes the loss of vesicular [^{14}C]-5-HT during the amine uptake process. In terms of the amounts of labelled material accumulated, 5-HT appears to be the preferred substrate, with dopamine second. All amines examined produced sizable non-thrombin-releasable (cytoplasmic) pools, although this fraction (expressed as a percentage of the total labelled material inside the platelet) was the smallest with octopamine.

The amount of total [^{14}C]-5-HT lost from *platelets* during the accumulation of the different amines varied from <2% to 5% of the pretreatment [^{14}C]-5-HT content for all amines except for [^{3}H]-5-HT, which produced greater (up to 22%) losses of [^{14}C]-5-HT. The amount of [^{14}C]-5-HT lost from *vesicles,* in contrast, ranged from <2% to almost 30% of the pretreatment content for all amines including 5-HT. Since the amount of [^{14}C]-5-HT in the vesicles prior to ^{3}H-amine addition represents only 1–5% of the total endogenous 5-HT content, and is released by thrombin to the same extent as endogenous 5-HT and the total dense-body pool, it can serve as a marker for the behaviour of the endogenous (unlabelled) 5-HT (Costa & Murphy, 1976; Costa *et al.,* 1977a). Thus, the loss of relatively small amounts of [^{14}C]-5-HT from a given compartment indicates that from 20–100 times as much total 5-HT is actually being displaced. The relatively greater amount of [^{14}C]-5-HT lost from vesicles than from the platelet would appear to be explained by material displaced from vesicles which accumulates in platelet cytoplasm.

The data presented in Table 1 raised two questions which we attempted to address by another series of experiments. Data from these experiments are presented in Tables 2–5. (1) What is the nature of the ^{3}H-labelled material in the cytoplasmic pool? Could, for example, this represent an aldehyde or acid amine metabolite, perhaps bound in some fashion to cytoplasmic protein? (2) What is the significance of the loss of [^{14}C]-5-HT from vesicles? Can, for example, 5-HT lost from vesicles during the addition of dopamine or tyramine re-enter the vesicular compartment?

Table 2 indicates that similar cytoplasmic and vesicular pools of 5-HT, dopamine, and tyramine were formed after 10 min of incubation with these ^{3}H-amines, regardless of whether platelet MAO was inhibited or not. Deprenyl appeared to inhibit slightly the uptake of [^{3}H]-5-HT, although the uptake of dopamine and tyramine appeared unchanged. It thus seems likely that cytoplasmic radioactivity does not represent oxidatively deamineated metabolites from these amines.

Tables 3 and 4 show the effects of extracellular and intracellular dopamine and tyramine on the uptake of [^{3}H]-5-HT. Over a 10 s period, neither amine at concentrations 10-fold and 100-fold higher than [^{3}H]-

Table 1 Measurement of total and vesicular ^{3}H-amine concentrations and the loss of vesicular [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) following a 30 min incubation with 10^{-6} M extracellular amine

	[^{3}H]-5-HT		[^{3}H]-dopamine		[^{3}H]-tyramine		[^{3}H]-octopamine		[^{3}H]-tryptamine	
	Total	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*
^{3}H-amine accumulated (mol/platelet × 10^{18})	110.5 ±3.71	88.47 ±1.00	21.14 ±1.09	13.38 ±0.29	7.04 ±0.35	2.55 ±0.16	3.48 ±0.16	3.10 ±0.15	5.17 ±0.63	2.11 ±0.21
[^{14}C]-5-HT lost* (mol/platelet × 10^{18})	0.98 ±0.09	1.27 ±0.05	<0.10 to 0.37†	<0.10 to 1.81†	<0.10†	0.52 ±0.15	<0.10 to 0.15†	0.65 ±0.18	0.28 ±0.02	0.99 ±0.26

Values are mean ± s.e. mean.

* Platelet storage vesicles contained 5.39 × 10^{-18} mol/platelet of [^{14}C]-5-HT before incubation.

† Amounts of [^{14}C]-5-HT less than 0.10 × 10^{-18} mol/platelet could not be measured.

Table 2 Measurement of total and vesicular amine concentrations in control and deprenyl-treated platelets following a 10 min incubation with 10^{-6} M extracellular amine

	Amount of ^{3}H-amine present (mol/platelet × 10^{18})					
	[^{3}H]-5-HT		*[^{3}H]-dopamine*		*[^{3}H]-tyramine*	
Pre-treatment	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*
None (control)	32.69 ± 1.15	23.30 ± 2.07	5.448 ± 0.188	1.765 ± 0.686	1.549 ± 0.112	0.117 ± 0.054
Deprenyl (10^{-4} M)	29.36 ± 0.32*	20.58 ± 2.61	5.362 ± 0.297	1.916 ± 0.373	1.462 ± 0 259	0.318 ± 0.176

Values are mean ± s.d.

* Significantly less than control value, $P < 0.005$, Student's *t* test.

Table 3 Lack of effect of various extracellular concentrations of tyramine and dopamine on the initial rate of uptake of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) into human platelets

Amine added with [^{3}H]-5-HT	*Amount of [^{3}H]-5-HT in platelets (mol/platelet per s × 10^{21})*
None	1.84 ± 0.34
Tyramine	
10^{-6} M	2.08 ± 0.35
10^{-5} M	1.91 ± 0.07
Dopamine	
10^{-6} M	1.99 ± 0.05
10^{-5} M	2.11 ± 0.20

Values are mean ± s.d.

Table 4 Effect of increasing concentrations of cytoplasmic and vesicular [^{14}C]-dopamine and [^{14}C]-tyramine on the uptake of exogenous [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT)

	Amount of [^{3}H]-5-HT accumulated in control, [^{14}C]-dopamine or [^{14}C]-tyramine prelabelled preparations after incubation for 1 min (mol/platelet × 10^{20})		*Amount of [^{14}C]-dopamine or [^{14}C]-tyramine accumulated after incubation for 10 min or 30 min (mol/platelet × 10^{19})*	
	Total	*Vesicular*	*Total*	*Vesicular*
Control	1.493 ± 0.013	1.081 ± 0.281	0	0
[^{14}C]-dopamine (10^{-5} M)				
10 min	1.507 ± 0.054	1.097 ± 0.186	11.735 ± 0.238	7.299 ± 0.390
30 min	1.633 ± 0.143	1.132 ± 0.202	33.590 ± 0.848	20.037 ± 1.702
[^{14}C]-tyramine (10^{-5} M)				
10 min	1.558 ± 0.111	1.396 ± 0.490	3.807 ± 0.132	0.987 ± 0.111
30 min	1.622 ± 0.095	1.217 ± 0.207	7.336 ± 0.134	1.673 ± 0.112

Values are mean ± s.d.

5-HT, appeared to have a significant effect on the movement of 10^{-7} M [^{3}H]-5-HT across the plasma membrane. Similarly, over a 1 min period, the amount of ^{3}H-labelled material (initial concentration 10^{-8} M) accumulating in platelet cytoplasm and vesicles was not significantly affected by either dopamine or tyramine in both vesicles and cytoplasm. It is of interest that [^{3}H]-5-HT uptake was unchanged in the face of cytoplasmic and vesicular ^{14}C-amine concentrations varying over a 2- to 3-fold range. Under these experimental conditions, the amount of [^{3}H]-5-HT moving into the vesicles during a 1 min period was approximately 100-fold less than the total amount of [^{14}C]-dopamine or tyramine present in the cytoplasm.

Table 5 indicates that the presence of cytoplasmic 5-HT significantly lowered the total amount of new 5-HT entering platelets and vesicles over a 1 min period. This experiment, it should be noted, is not strictly comparable to that presented in Table 4, since Incubation A and B platelets contained large cytoplasmic pools of both labelled and unlabelled (endogenous) 5-HT, the latter displaced from vesicles during the addition of labelled material to this compartment.

Discussion

The experiments described here focus on the uptake and intra-platelet distribution of 5-HT and several other biogenic amines. Initially, we measured net accumulation of amines and concomitant changes in the content of a tracer amount of vesicular [^{14}C]-5-HT. The initial data suggested some hypotheses which we then investigated with subsequent experiments.

(1) Under the experimental conditions employed here, 5-HT appears to be the preferred substrate for uptake into both platelets and vesicles, although dopamine is a surprisingly good second-place contender. Octopamine is also of interest in that a larger percentage of the total accumulated by the platelet is sequestered in vesicles (90%), as compared to the range found for other amines (from 36% to 87%). This finding is consistent with a previous report that some of the octopamine present in human platelets appears to be located in platelet vesicles (Murphy, Cahan & Molinoff, 1975).

Table 5 Effect of cytoplasmic 5-hydroxytryptamine on the uptake of exogenous [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT)

	Amount of [^{3}H]-5-HT accumulated during a 1 min incubation (mol/platelet × 10^{21}) Total	Vesicular
Control	9.936 ± 0.185	5.405 ± 0.524
Incubation A*	6.427 ± 0.461	1.330 ± 0.440
Incubation B†	5.796 ± 0.398	0.721 ± 0.131

Values of mean ± s.d.

* Platelets contained 1.163×10^{-18} mol/platelet of labelled 5-HT in a cytoplasmic (non-vesicular) compartment.

† Platelets contained 2.176×10^{-18} mol/platelet of labelled 5-HT in a cytoplasmic compartment.

(2) The cytoplasmic pools of all amines examined are relatively large. The labelled material in this compartment does not appear to represent acid or aldehyde oxidative metabolites, since a pool of similar size forms even after treatment of intact platelets with the irreversible MAO-inhibitor deprenyl (Donnelly & Murphy, 1977). For these particular experiments, we chose to examine 5-HT, dopamine, and tyramine, since dopamine and tyramine are rapidly-deaminated substrates for the deprenyl-sensitive MAO-B found in human platelets (Donnelly & Murphy, 1977).

(3) Some of the data presented here suggest that the rate of entry of amines into vesicles may not be limited by the rate of entry of new amine into the cytoplasm. As shown in Table 4, a considerable increase in the cytoplasmic pools of dopamine and tyramine can occur between 10 and 30 min of incubation (from 5 to 13×10^{-19} mol/platelet for dopamine, and from 3 to 5×10^{-19} mol/platelet for tyramine). Despite this increase, the entry of dopamine into vesicles appears linear, while the vesicular pool of tyramine does not increase in size in parallel with the cytoplasmic pool. We can speculate that the rate of entry of a given amine into vesicles with 10^{-5} M extracellular amine concentrations may be determined by the rate of transport across the vesicular membrane, or by the availability of intra-vesicular binding sites for that amine, rather than by the rate at which material is transported across the plasma membrane.

(4) Taken together, our data are compatible with the hypothesis that once displaced from vesicles into platelet cytoplasm, 5-HT is not subsequently re-accumulated by the vesicles. Newly-added cytoplasmic 5-HT, in contrast, is a substrate for vesicular accumulation even in the absence of extracellular 5-HT, a property incidentally not shared by newly-added cytoplasmic dopamine or tyramine. Vesicular [^{14}C]-5-HT, and presumably endogenous 5-HT, is lost from the platelet during the net addition of new [^{3}H]-5-HT. If the incoming and outgoing 5-HT pools were identical, mixing within the cytoplasm would prevent any net loss of [^{14}C]-5-HT (see also Costa *et al.*, 1977a; Costa, Fauci & Wolff, 1976). In addition, neither dopamine nor tyramine appears to inhibit the entry of new [^{3}H]-5-HT into platelet cytoplasm or vesicles. Thus re-uptake of vesicular [^{14}C]-5-HT should occur if the [^{14}C]-5-HT behaved in a fashion similar to newly-added [^{3}H]-5-HT.

These experiments suggest that the dynamics of the platelet amine uptake and storage system are quite complex, despite negligible contributions from *de novo* amine synthesis (Murphy & Kopin, 1972). The data are difficult to interpret in terms of any simple model, despite the fact that these experiments provided a means to explore the platelet under conditions where metabolism appeared unimportant, and where accurate assessment of cytoplasmic and vesicular pools of both newly-added and vesicular 5-HT was possible. In the light of our experience, obtaining and interpreting amine-flux data with other aminergic systems, such as synaptosomes, may present more problems than previously anticipated.

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(Received January 25, 1977.)

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THE INHIBITION OF HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS BY NON-STEROID ANTI-INFLAMMATORY DRUGS AND ITS REVERSAL BY CALCIUM

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1 The non-steroid anti-inflammatory drugs, indomethacin, flufenamate and meclofenamate, inhibited the release of histamine from rat peritoneal mast cells induced by pharmacological or immunological challenge *in vitro*.

2 Anti-inflammatory steroids had little effect on histamine release from the mast cells.

3 Th inhibition of histamine release by the aspirin-like drugs was not prevented by incubation with glucose, unlike the inhibition of 2,4,dinitrophenol or antimycin-A. This suggests that the non-steroid anti-inflammatory compounds do not act by preventing the energy production from oxidative metabolism, required for histamine release.

4 The inhibition of the calcium ionophore A23187-induced histamine release by the aspirin-like drugs was reversed by an increase in the calcium concentration of the incubation medium.

5 The results suggest that the non-steroid anti-inflammatory compounds inhibit histamine release by actions on calcium influx into the mast cell, or on calcium mobilization or utilization within the mast cell.

Introduction

Primary prostaglandins of the E series and their analogues can inhibit the release of histamine from rat peritoneal mast cells *in vitro* induced by pharmacological or immunological challenge (Loeffler, Lovenberg & Sjoerdsma, 1971; Kaliner & Austen, 1974; Thomas & Whittle, 1976). To define a possible role for endogenous mast cell prostaglandins in the histamine release process, the effects of non-steroid anti-inflammatory agents, known to inhibit prostaglandin biosynthesis (Vane, 1971), were investigated. However, these drugs, like the primary prostaglandins studied, were found to reduce histamine release from the mast cells (Thomas & Whittle, 1976). In the present paper, possible mechanisms by which these aspirin-like drugs inhibit histamine release have been investigated.

A preliminary account of this work has been presented to the British Pharmacological Society (Whittle, 1976).

Methods

Mast cells were obtained by lavage of the rat peritoneal cavity with a modified buffered medium (pH7) containing bovine serum albumin (0.1% w/v). Unless otherwise stated, the lavage solution had the following composition (mM):NaCl 154, KCl 2.7, $CaCl_2$ 0.9, buffered with Na_2HPO_4 4.5 and KH_2PO_4 2.2. For experiments using higher calcium concentrations, the concentration of the phosphate buffers was reduced (by 50%) to avoid precipitation. In studies with 'calcium-free' medium, analar water (BDH; containing less than 1 part per million calcium) was used to dissolve the above salts.

Male Wistar rats (300–400 g, body weight) were lightly anaesthetized with ether, decapitated and exsanguinated, and the buffered-albumin medium (14 ml) slowly injected intraperitoneally. After a 2 min massage of the rat abdomen, the peritoneal cavity was opened by a mid-line incision and the fluid (10 ml) was transferred to a plastic tube and centrifuged (approximately 200 *g* for 3 minutes). The supernatant was discarded and the cell pellet resuspended in the buffer-albumin medium (2.5 ml). Aliquots (0.1 ml) were distributed to small plastic tubes containing albumin-free buffered solution (0.1 ml) and the drug under investigation (0.1 ml). Following the pre-incubation (at 22°C or 37°C where appropriate), the histamine-liberating agent (0.1 ml) was added and the cells further incubated. The incubation was terminated by addition of the cold albumin-free buffered solution (0.5 ml at 4°C) and the tubes centrifuged (700 *g* for 5 minutes).

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Aliquots (0.1 ml) of the supernatant were transferred to plastic vials (Sterilin Ltd.) containing distilled water (2ml) and the histamine content was assayed by alkaline condensation (0.4 ml 1 N NaOH) with O-phthaladehyde (10 mg/ml in methanol; 0.1 ml) followed by acidification (0.2 ml 3 HCl after 4 min) and measurement of the fluorescence (excitation 350 nm, emission 440 nm; Shore, Burkhalter & Cohn, 1959). Histamine concentrations determined by this fluorometric method were in good agreement with values obtained by bioassay on the superfused guinea-pig ileum (5 experiments). In the concentrations employed in the study, none of the drugs interfered with this fluorometric histamine assay.

In any experiment using the batch of mast cells from one rat, each experimental sequence and drug treatment was performed at least in duplicate, and each experiment was repeated with 5 or more batches of mast cells.

The number of mast cells in the resuspended cell pellet was counted in a haemocytometer, following staining of a sample of the cell suspension with a toluidine-blue solution (Bray & Van Arsdel, 1961).

Rats were sensitized by subcutaneous administration of the antigen, either egg albumin (50 mg) or inactivated horse serum (0.5 ml), together with administration of *B. pertussis* suspension (0.5 ml s.c.; 4×10^{10}/ml, Wellcome Reagents Ltd.). The rats were re-injected with the antigen alone on the second day, and the animals used 14–28 days later.

Drugs

The histamine liberator compound 48/80, Triton X-100 (Sigma Chemical Co.) and *n*-decylamine (Aldrich Chemical Co.) were stored in distilled water (4°C) and freshly diluted to the desired concentration with albumin-free buffered medium (at least a 100-fold dilution was used to avoid possible artifacts). Adenosine triphosphate (Sigma Chemical Co.), crude phospholipase-A (*Vipera russellii* venom, Koch-light Labs.) and the antigens, inactivated horse serum (Wellcome Reagents Ltd.) or egg albumin (fraction *V*, Sigma Chemical Co.) were dissolved directly in the buffered medium. The non-steroid anti-inflammatory agents indomethacin (Merck, Sharp & Dohme, Ltd.), meclofenamic acid and flufenamic acid (Parke Davis & Co.) were dissolved directly in the buffered medium; the use of sodium bicarbonate solution to increase solubility was avoided to obviate any possible interference. The anti-inflammatory steroids dexamethasone sodium phosphate (Merck, Sharp & Dohme, Ltd.) and betamethasone sodium phosphate (Glaxo) were dissolved in a similar fashion, as were the other compounds, 2,4,dinitrophenol and glucose (BDH).

Antimycin-A (Sigma Chemical Co.) and the ionophore-A23187 (Lilly Research Centre Ltd.) were stored in absolute ethanol or methanol and diluted (at least 100-fold) with the buffered solution immediately before use; the final alcohol content so obtained had no detectable effect on the mast cells.

Statistical analysis

Results are expressed as mean ± standard error of the mean, where (*n*) represents the number of values in the group. The significance of difference between the grouped data was evaluated by Student's *t* test for paired or unpaired values, where appropriate. $P < 0.05$ was taken as significant.

Results

Characteristics of mast cell suspensions

The number of mast cells in the resuspended mixed peritoneal cell pellet was $3.5–6 \times 10^{-6}$/2.5 ml ($n = 56$) with a total histamine content (following cell disruption with perchloric acid, 0.4 M) of 30–60 µg/2.5 ml, i.e. 8.3 ± 0.4 µg histamine/10^6 mast cells.

Histamine release from mast cells

The low resting release of histamine (1–3% of the total histamine content) from the mast cells could be readily increased in a dose-dependent manner by incubation with the histamine liberator, compound 48/80 (0.05–1 µg/ml, 5 min at 22°C or 37°C) or adenosine triphosphate (50–400 µg/ml; 5 min at 37°C for optimal release). The release induced by the crude phospholipase-A from *Vipera russellii* (2.5–20 µg/ml; 10 min at 37°C for optimal release) may be related to the presence of a lytic factor since phospholipase-A from three other sources had little histamine-releasing activity (Brain, Lewis & Whittle, 1977). The dose-release relationship with the calcium ionophore A23187 (0.125–0.5 µg/ml; 5 min at 22°C or 37°C) was relatively steep, as was that observed with non-specific liberators, Triton X-100 (50–200 µg/ml) and *n*-decylamine (20–80 µg/ml).

Inhibition of histamine release by non-steroid anti-inflammatory agents

Samples of cell suspension were pre-incubated at room temperature with indomethacin (0.5–80 µg/ml for

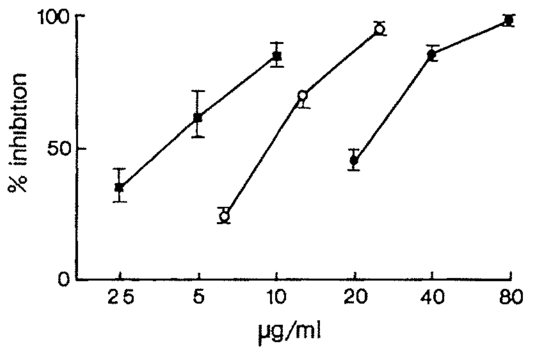

Figure 1 Inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells by non-steroid anti-inflammatory drugs: (■) meclofenamate; (○) flufenamate, (●) indomethacin. Results are shown as the mean of at least 5 experiments for each group. Vertical lines show s.e. mean.

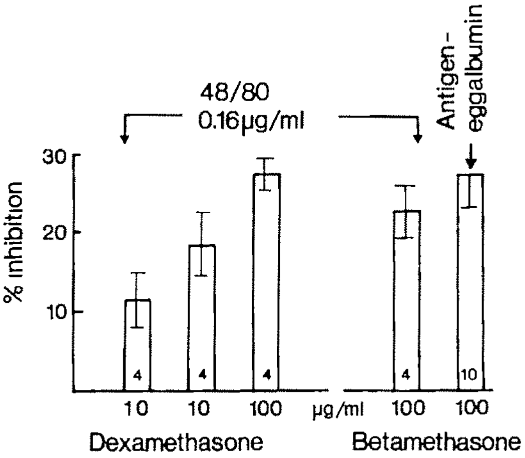

Figure 2 Effects of anti-inflammatory steroids on the release of mast cell histamine induced by compound 48/80 and the antigen, egg albumin. Results are shown as mean of at least 4 experiments in each group. Vertical lines show s.e. mean.

5 min), followed by addition of compound 48/80 (0.16 µg/ml) in a dose giving 30–50% release of the total histamine in the mast cells in the control experiments. The lower concentrations of indomethacin (0.6–5 µg/ml) had no effect on the histamine release, whereas higher concentrations (10–80 µg/ml) caused a dose-dependent inhibition of release (Figure 1). Indomethacin (0.6–80 µg/ml) had no significant effect on the low resting histamine release (5 experiments).

In a further 6 experiments, indomethacin (20 µg/ml) gave a parallel displacement of the dose-release curve with compound 48/80 (0.1–0.8 µg/ml). In other experiments, the effects of temperature and the duration of pre-incubation with indomethacin (10 µg/ml) were studied. Inhibition of 48/80-induced release was comparable at either 22°C or 37°C (23 ± 6% and 21 ± 7% inhibition respectively, $n=5$) and increasing the period of pre-incubation from 5 min to 20 min had little effect on the degree of inhibition. For subsequent experiments, a 5 min pre-incubation period was therefore used.

The non-steroid anti-inflammatory agents meclofenamic acid and flufenamic acid also caused a similar dose-dependent inhibition of 48/80-induced histamine release (Figure 1); the doses causing 50% inhibition of submaximal histamine release (ID_{50}) were 3.5 and 9.0 µg/ml respectively, compared with an ID_{50} of 21.5 µg/ml for indomethacin. As shown in Table 1, pre-incubation with the aspirin-like drugs also inhibited submaximal histamine release induced by adenosine triphosphate (200 µg/ml), by crude phospholipase-A (5 µg/ml), by the calcium ionophore A23187 (0.3 µg/ml) and by antigenic challenge with egg albumin (1000 µg/ml), and also with inactivated horse serum (1 in 500 final dilution with buffered medium). In these experiments, the aspirin-like drugs were more potent as inhibitors of ionophore A23187-induced histamine release (Table 1). In contrast, these drugs had no consistent effect on non-specific histamine liberation by the surface-active agents, Triton X-100 (0.01%) or *n*-decylamine (25 µg/ml).

Table 1 Inhibition of histamine release from rat peritoneal mast cells

Histamine liberator	*(µg/ml)*	*Indomethacin*	*Meclofenamate*
Compound 48/80	(0.16)	21.5	3.5
Ionophore-A23187	(0.3)	7.0	1.5
Phospholipase-A	(5.0)	13.5	2.0
Adenosine triphosphate	(200)	10.0	3.0
Antigen-egg albumin	(1000)	13.0	6.5

The concentration of inhibitor (µg/ml) giving 50% reduction of histamine release (ID_{50}), following 5 min pre-incubation with mast cells, was determined from at least 5 experiments. Control histamine release, with the concentration of each liberator shown, was 30–50% of total histamine content of the cells.

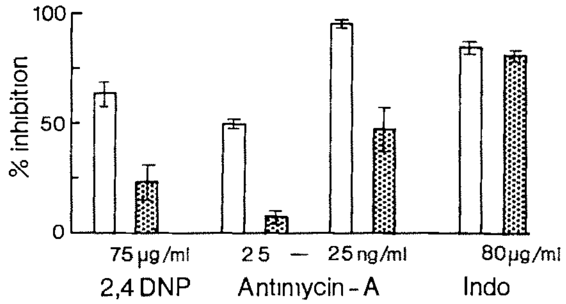

Figure 3 Effects of pre-incubation with glucose (5 mM for 10 min) on the inhibition of compound 48/80-induced histamine release by 2,4,dinitrophenol (2,4 DNP), antimycin-A and indomethacin (Indo). Stippled columns—preincubation with glucose; open columns—no preincubation with glucose. Results are the mean of 5 experiments in each group. Vertical lines show s.e. mean.

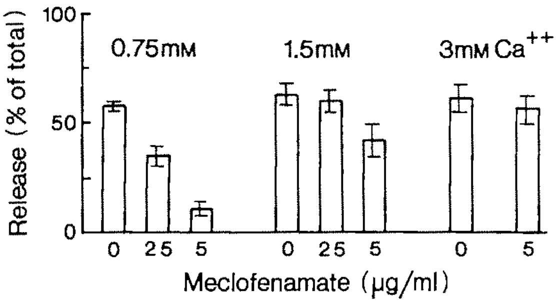

Figure 4 Effects of calcium concentration (0.75–3 mM) on the inhibition of ionophore A23187-induced histamine release by meclofenamate. Results, shown as histamine release, % of total histamine in the mast cells, are the mean of at least 4 experiments in each group. Vertical lines show s.e. mean

Effects of anti-inflammatory steroids

As shown in Figure 2, the anti-inflammatory steroids dexamethasone (1–100 µg/ml) and betamethasone (1–100 µg/ml) in high concentrations, caused a small inhibition of submaximal histamine release induced by compound 48/80 and the antigen, egg albumin (4 experiments in each group). In other experiments, the histamine release induced by crude phospholipase-A was also inhibited by dexamethasone (by 39.6 ± 4.3%, $n = 7$; with 100 µg/ml).

Effects on oxidative metabolism

The inhibitory effects of the aspirin-like drugs on histamine release were compared with those of drugs known to interfere with oxidative metabolism. Submaximal histamine release induced by compound 48/80 (0.2 µg/ml) was inhibited in a dose-dependent manner by 2,4,dinitrophenol (50–100 µg/ml) or antimycin-A (2.5–25 ng/ml) (Figure 3). Pre-incubation of the mast cells with medium containing glucose (1–5 mM) for 10 min, significantly ($P < 0.01$, in each case) reversed this inhibition (Figure 3). In contrast, glucose pre-incubation had no significant ($P < 0.05$) effect on the inhibition of histamine release by indomethacin (20–80 µg/ml) or meclofenamate (5–20 µg/ml).

In a further 5 experiments, the inhibition of ionophore A23187 (0.3 µg/ml)-induced histamine release by antimycin-A (0.6 ng/ml) was reduced by pre-incubation with glucose-(1 mM)-containing medium (from 39 ± 9% to 15 ± 10% inhibition; $P < 0.01$). The inhibition with a higher dose of antimycin-A (6 ng/ml) was likewise reduced by pre-incubation in medium containing glucose (from 90 ± 3% to 71 ± 5% inhibition; $n = 5$). However, there was no marked effect on the inhibition by meclofenamate (5 µg/ml; 51 ± 3% and 43 ± 5% inhibition, with and without glucose respectively).

Another difference between the two classes of compound was that whereas the dose of the 'metabolic inhibitor' required to prevent histamine release could vary greatly (up to 10-fold) from month to month, the inhibitory concentrations of the aspirin-like drugs remained constant in experiments carried out over one year.

Effects of calcium concentration

Initial experiments confirmed previous observations (Foreman, Mongar & Gomperts, 1973) that the release of histamine by ionophore A23187 was dependent not only on the dose (0.1–0.5 µg/ml) but also on the calcium concentration (0.5–3 mM) of the incubation medium. Optimal histamine release, with a submaximal dose of the ionophore (0.3 µg/ml), was obtained with a calcium concentration of 0.75 mM; an increase in the calcium concentration up to 3 mM did not elevate the release further (Figure 4). The dose-dependent inhibition of histamine release by meclofenamate (2.5–5 µg/ml) or indomethacin (10–40 µg/ml) was related to the calcium concentration of the incubation medium (Figure 4). In four experiments, the inhibition of release by meclofenamate (5 µg/ml) was significantly reduced (from 81 ± 3% to 8 ± 4% inhibition, $P < 0.001$) when the calcium concentration was increased from 0.75 mM to 3 mM (Figure 4). Similarly, the inhibition of ionophore-induced histamine release by indomethacin (15 µg/ml) was reduced (from 62 ± 4 to 21 ± 4%, $P < 0.01$) by increasing the calcium concentration from 0.75 mM to 1.5 mM.

This reversal by calcium was not a non-specific

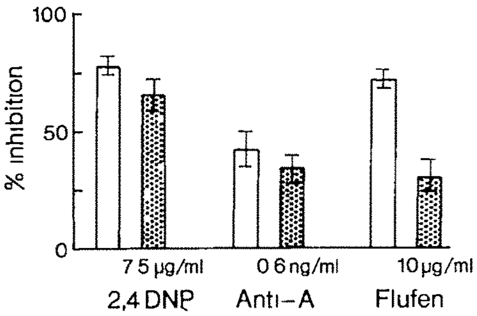

Figure 5 Effects of calcium concentration (open columns 1 mM; stippled columns 2 mM) on the inhibition of ionophore A23187-induced histamine release by 2,4,dinitrophenol (2,4 DNP), antimycin-A (Anti-A) and flufenamate (Flufen). Results are the mean of 5 experiments in each group. Vertical lines show s.e. mean.

action since in the 5 experiments shown in Figure 5, the inhibition of ionophore-induced histamine release by 2,4,dinitrophenol (7.5 µg/ml) or antimycin-A (0.6 ng/ml) was not significantly altered, whereas the inhibition by flufenamate (10 µg/ml) was considerably reduced by the increased calcium concentration (from 1 to 2 mM; $P<0.001$).

In an initial series of experiments to investigate the effects of calcium lack on histamine liberation was compound 48/80, the histamine release induced in 'calcium-free' buffered medium was also inhibited by indomethacin (45 ± 8%, $n=7$; with 40 µg/ml) and meclofenamate (41 ± 4%, $n=4$; with 5 µg/ml).

Discussion

Prostaglandins of the E series inhibit anaphylactic histamine release *in vitro* from rat peritoneal mast cells (Kaliner & Austen, 1974; Thomas & Whittle, 1976) and from chopped human lung (Walker, 1973). This *in vitro* action which follows incubation with exogenous prostaglandins could reflect a pathophysiological modulator role for prostaglandins *in vivo* during anaphylaxis. The finding that low concentrations of non-steroid anti-inflammatory agents which inhibit prostaglandin formation (Vane, 1971), can augment the release of histamine and slow reacting substance of anaphylaxis (SRS-A) from isolated perfused lung of guinea-pig following challenge with antigen (Engineer, Piper & Sirois, 1976), again suggests that prostaglandins can modulate the anaphylactic release of histamine and SRS-A. In contrast, in the present study, low concentrations of the aspirin-like drugs had no effect on histamine release from rat mast cells, suggesting that endogenous mast-cell prostaglandins are not involved in the histamine release process. It is possible, therefore, that in the perfused lung, the prostaglandins are released from cells other than those which release histamine or SRS-A. These latter mediators, released during challenge, may lead to prostaglandin generation, which could then act through a 'negative feed-back mechanism' to modulate the further release of the mediators.

The findings that non-steroid anti-inflammatory drugs could inhibit the release of histamine from rat peritoneal mast cells induced by a variety of stimuli support the observations that indomethacin reduces rat mast cell degranulation (Taylor, Francis, Sheldon & Roitt, 1974) and histamine release from human lung *in vitro* (Walker, 1973). In earlier studies, several other aspirin-like compounds, such an phenylbutazone or sodium salicylate were found to inhibit anaphylactic histamine release *in vitro* from rat skin (Mota & Ishii, 1960), from rat mast cells (Norn, 1965) and from chopped guinea-pig lung (Mongar & Schild, 1957), although high concentrations were used. However, inhibition of compound 48/80-induced histamine release from rat mast cells by non-steroid anti-inflammatory drugs, in a similar concentration range as in the present study, has been reported (Yamasaki & Saeki, 1967).

In the present work, the doses of aspirin-like drugs required to inhibit histamine release were somewhat greater than those which should be required to inhibit prostaglandin biosynthesis, and thus these effects are probably not related to effects on prostaglandin formation. However, it should be pointed out that the presence of albumin in the incubation medium would be expected to reduce the concentration of free aspirin-like drugs which are known to bind avidly to albumin. The ability of these drugs to inhibit histamine release from mast cells following challenge with several pharmacological agents and antigen, but not that induced by non-specific surface-active agents, suggests an action on some common mechanism involved in the release process.

Since agents which can elevate cyclic adenosine 3′,5′-monophosphate (cyclic AMP) levels in mast cells have been shown to inhibit histamine release (Loeffler *et al.*, 1971; Taylor *et al.*, 1974; Kaliner & Austen, 1974), it is possible that the non-steroid anti-inflammatory compounds, by virtue of their ability to inhibit phosphodiesterase (Newcombe, Thanassi & Ciosek, 1974) were acting by a similar mechanism. It is also of interest that in the present study, the steroid anti-inflammatory agents caused a small inhibition of histamine release induced by compound 48/80 and immunological challenge, as was found for antigen-evoked histamine release from human skin (Greaves & Plummer, 1974), since it has been suggested, from studies on human lymphocytes, that such compounds can likewise increase cyclic AMP levels by phosphodiesterase inhibition (Lavin, Rachelefsky & Kaplan, 1975). However, the aspirin-like drugs were very potent inhibitors of histamine release induced by the ionophore A23187, suggesting that other mechanisms may also operate; an increase in cyclic AMP levels, either by phosphodiesterase inhibitors or dibutyryl analogue of cyclic AMP, is not thought to prevent ionophore-induced histamine release (Garland & Mongar, 1976).

It was possible that these non-steroid anti-inflammatory compounds could affect the processes of oxidative metabolism involved in histamine release. Several such compounds have been shown, for example, to uncouple oxidative phosphorylation *in vitro* in rat liver mitochondria (Whitehouse, 1964). However, in the present study, addition of glucose to the incubation medium reversed the inhibitory effects of 2,4,dinitrophenol (which uncouples oxidative phosphorylation) or antimycin-A (which acts on the cytochrome system) by promoting anaerobic glycolysis (Peterson, 1974; Diamant, Grosman, Stahl Skov & Thomle, 1974), but had no effect on the inhibition by meclofenamate or indomethacin. This agrees with previous observations that glucose had little effect on the inhibition of histamine release by aspirin-like drugs compared with its effects on the inhibition by several metabolic inhibitors (Yamasaki & Saeki, 1967). The present findings strongly suggest, therefore, that these non-steroid anti-inflammatory drugs, in the concentrations used, do not interfere with the energy production from oxidative metabolism required for the histamine release process.

The finding that increased calcium concentrations could overcome the inhibition of ionophore-stimulated histamine release by aspirin-like drugs suggests that they act on a calcium-dependent stage of the release process. The entry of calcium into the mast cell is known to be an important stage in the release process (Foreman *et al.*, 1973) and certain 'anti-allergic' drugs, such as disodium cromoglycate, which inhibit histamine release, may act by preventing this calcium movement (Foreman, Mongar, Gomperts & Garland, 1975). It is therefore pertinent that several aspirin-like drugs have been shown to interfere with the uptake and binding of calcium in various tissues (Northover, 1973). Thus, inhibition of histamine release, following challenge with a variety of agents, by the non-steroid anti-inflammatory drugs *in vitro* could reflect actions on calcium influx into the mast cell. However, the process of calcium entry into the mast cell following ionophore-stimulation appears to differ from that following stimulation with antigen, in that it is not altered by cromogylcate or phosphodiesterase inhibitors (Foreman, Hallett & Mongar, 1977); and histamine release by compound 48/80 may not depend on the movement of extracellular calcium (see Cochrane & Douglas, 1974). It is therefore possible that aspirin-like drugs affect calcium mobilization or utilization within the mast cell. This is further supported by the present observations that the histamine release induced by compound 48/80 in calcium-free medium, which is thought to involve movement of intracellular calcium (Cochrane & Douglas, 1974) could also be inhibited by the aspirin-like drugs.

It is not known whether such actions could occur *in vivo* with anti-inflammatory doses of the aspirin-like drugs. However, it may be expedient to be aware of such a potential interaction between non-steroid anti-inflammatory agents and calcium when interpreting the actions of such drugs *in vitro* on any calcium-sensitive system.

This work was supported by the M.R.C. The calcium ionophore A23187 was kindly supplied by the Lilly Research Centre. We thank R.U. Thomas for excellent technical assistance.

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(Received January 25, 1977.)

Br. J. Pharmac. (1977), **61**, 237–242

THE RELATIONSHIP BETWEEN ENERGY METABOLISM AND THE ACTION OF INHIBITORS OF HISTAMINE RELEASE

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1 Dextran-induced release of histamine from rat mast cells was inhibited equally in complete and glucose-free Tyrode solution by doxantrazole (0.03–3 µmol/l), theophylline (0.1–3 mmol/l) and dicumarol (0.01–10 µmol/litre).

2 Doxantrazole (3 µmol/l), theophylline (3 mmol/l) and dicumarol (10 µmol/l) did not reduce the adenosine 5′-triphosphate (ATP) content of mast cells in glucose-free medium. Higher concentrations of dicumarol (56–100 µmol/l) markedly reduced the cellular ATP content. This reduction was reversed by glucose.

3 Papaverine was a more potent inhibitor of histamine release from mast cells incubated in glucose-free solution than in complete Tyrode solution (dose-ratio = 20). Like antimycin A (1 µmol/l), papaverine (3 µmol/l) caused a depletion of mast cell ATP that was greater in the absence (85%) than in the presence (25%) of extracellular glucose.

4 These results suggest that dicumarol, like doxantrazole and theophylline, inhibits histamine release without affecting mast cell energy metabolism. In contrast, papaverine probably inhibits release by depleting ATP that is required for exocytosis.

5 Inhibition of histamine release by dibutyryl cyclic adenosine 3,5′-monophosphate (1–3 mmol/l) was significantly greater when cells were incubated in complete rather than in glucose-free medium.

Introduction

In addition to being dependent upon extracellular calcium (Foreman & Mongar, 1972a) the anaphylactic release of histamine from rat peritoneal mast cells has a requirement for metabolic energy, being supported by adenosine 5′-triphosphate (ATP) derived from either glycolytic or oxidative pathways. This conclusion is based on experiments with metabolic inhibitors and measurement of the cellular content of ATP (Peterson, 1974; Diamant, Norn, Fielding, Olsen, Ziebell & Nissen, 1974; Johansen & Chakravarty, 1975). Experiments using the divalent cation ionophore, A23187, which facilitates the passage of calcium across cell membranes, suggest that ATP is required for exocytosis at a stage following calcium entry into the cell (Foreman, Mongar & Gomperts, 1973).

The dextran-induced release of histamine from rat mast cells *in vitro* shares many properties with the anaphylactic reaction including calcium-dependence (Foreman & Mongar, 1972b), enhancement by phosphatidyl serine (Goth, Adams & Knoohuizen, 1971), desensitization and cross-desensitization with antigen (Foreman & Garland, 1974; Foreman, Garland & Mongar, 1976) and inhibition by antiallergic agents such as cromoglycate (Garland & Mongar, 1974). Furthermore, the reaction is diminished by metabolic inhibitors such as 2,4-dinitrophenol (Beraldo, Dias da Silva & Lemos Fernandes, 1962).

In recently described differential release experiments, several compounds were compared as inhibitors of histamine release induced by either dextran or the ionophore (A23187) (Garland & Mongar, 1976). Two compounds, papaverine and dicumarol, had effects that were not consistent with their generally accepted properties. Thus, papaverine which is known as a phosphodiesterase inhibitor, appeared to have the effect of a metabolic inhibitor while dicumarol, which is known to uncouple oxidative phosphorylation in some systems (Mahler & Cordes, 1967) had a profile of effect resembling cromoglycate rather than 2,4-dinitrophenol. Therefore, the present experiments were undertaken to examine the effects of these inhibitors of histamine release on energy metabolism in rat mast cells.

Methods

Histamine release was induced by dextran from mixed peritoneal cells of male Wistar rats (1–5% mast cells) prepared *in vitro* as described previously (Garland & Mongar, 1974). A 0.5 ml volume of a mixture of dextran and phosphatidyl serine (PS) was added to 0.5 ml cells preincubated for 30 min at 37°C in Tyrode solution with or without glucose (5.6 mmol/l); the final concentrations were dextran 6 mg/ml, PS 30 µg/ml. Where appropriate, drugs were included with dextran and PS in the releasing mixture. Histamine release was stopped after 5 min incubation at 37°C by the addition of 4 ml ice-cold calcium-free Tyrode and each sample was centrifuged at 250 *g* for 6 minutes. The supernatant was decanted, the pellet resuspended in 5 ml Tyrode and boiled for 10 min to release residual histamine. Released and residual histamine was assayed fluorimetrically by the method of Shore, Burkhalter & Cohn (1959) but omitting the extraction step as described by Loeffler, Lovenberg & Sjoerdsma (1971). The control histamine release from cells preincubated in complete medium was 35.7 ± 1.3% (mean ± s.e. mean of 45 observations) compared with 49.6 ± 1.6% from samples of the same cell suspensions incubated in glucose-free medium. This difference is consistent with the inhibition by glucose of dextran anaphylactoid reactions in rats described by Beraldo *et al.* (1962) and Poyser & West (1968). Inhibition of dextran-induced release from cells incubated in either glucose-free or complete medium was calculated by reference to the appropriate control release corrected for the spontaneous histamine release which was the same in each medium (6.7 ± 0.4%).

Measurement of ATP was made using cells isolated from recently killed male Sprague-Dawley rats (320–500 g) and fractionated by differential centrifugation through human serum albumin (Chakravarty & Zeuthen, 1965; Chakravarty, 1965). The mast cell fraction was washed twice to remove the excess albumin and suspended in Krebs-Ringer solution containing human serum albumin, 1 mg/ml (final pH 7.0–7.1). The purity of the mast cells was 96.4 ± 0.8% (mean ± s.e. mean of 12 experiments). Mast cells pooled from 1–5 rats were divided into aliquots containing 40,000–95,000 cells in a final volume of 0.5 ml. The cell suspensions were prewarmed in a 37°C water bath for 10 min and the incubation continued thereafter with different drugs for between 10 to 30 minutes. Samples without drugs and other appropriate controls were included. After incubation, the reaction was stopped by quickly adding double the volume of chilled perchloric acid (PCA) giving a final PCA concentration of 330 mmol/litre. Extraction and determination of the mast cell ATP content was performed as described previously with a specially constructed photometer to measure the peak initial flash from the firefly lantern luciferin-luciferase reaction (Johansen & Chakravarty, 1975). Each experiment was performed in either duplicate or triplicate. The ATP content of mast cells after incubation with drugs is given as a percentage of the control value which was 1.22 ± 0.04 pmol per 10^3 cells (mean ± s.e. mean of 12 experiments). There was no significant difference between the ATP levels in control cells incubated with or without glucose.

Dextran (mol. wt. 110,000) was supplied by Fisons as a 6% (w/v) solution in saline (NaCl 154 mmol/l). Phosphatidyl serine supplied by Koch-Light Ltd. was mixed with saline in a mechanical blender after evaporation of the chloroform-methanol solvent in a stream of nitrogen. Desiccated firefly tails (lanterns), ATP, antimycin A, dicumarol and N^6-O_2'-dibutyryl cyclic adenosine 3′,5′-monophosphate, sodium salt (dibutyryl cyclic AMP) were purchased from the Sigma Chemical Company. Theophylline and doxantrazole were supplied by the Wellcome Research Laboratories. Antimycin A was dissolved in ethanol and then diluted with Krebs-Ringer solution containing albumin 1 mg/ml. The final concentration of ethanol was 0.02% v/v. As shown previously ethanol even in a concentration of 2% v/v did not influence the ATP content of the mast cells (Johansen & Chakravarty, 1975).

The incubation media had the following compositions (mmol/l): Tyrode solution – NaCl 137, KCl 2.7; $MgCl_2$ 1.0, $CaCl_2$ 1.8, $NaHCO_3$ 12 and NaH_2PO_4 0.3; Krebs-Ringer solution – NaCl 141.9, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.0, Na_2HPO_4 2.5 and KH_2PO_4 0.6. Where appropriate, glucose was added to achieve a final concentration of 5.6 mmol/litre. All chemicals were of Analar quality.

Results

The dextran-induced release of histamine from rat peritoneal mast cells was inhibited by the antiallergic agent, doxantrazole, at concentrations of between 0.03–3 µmol/l added to cells concomitantly with the releasing stimulus (Figure 1a). This dose-related inhibition was identical in cells from the same original population but suspended in glucose-free rather than complete Tyrode solution. Inhibition of histamine release by theophylline, 0.1–3 mmol/l (Figure 1b) and dicumarol, 0.01–10 µmol/l (Figure 1c) was also unaffected by the glucose content of the preincubation medium. Figure 2a shows that dibutyryl cyclic AMP was slightly more potent when added to cells preincubated in glucose-containing medium. In

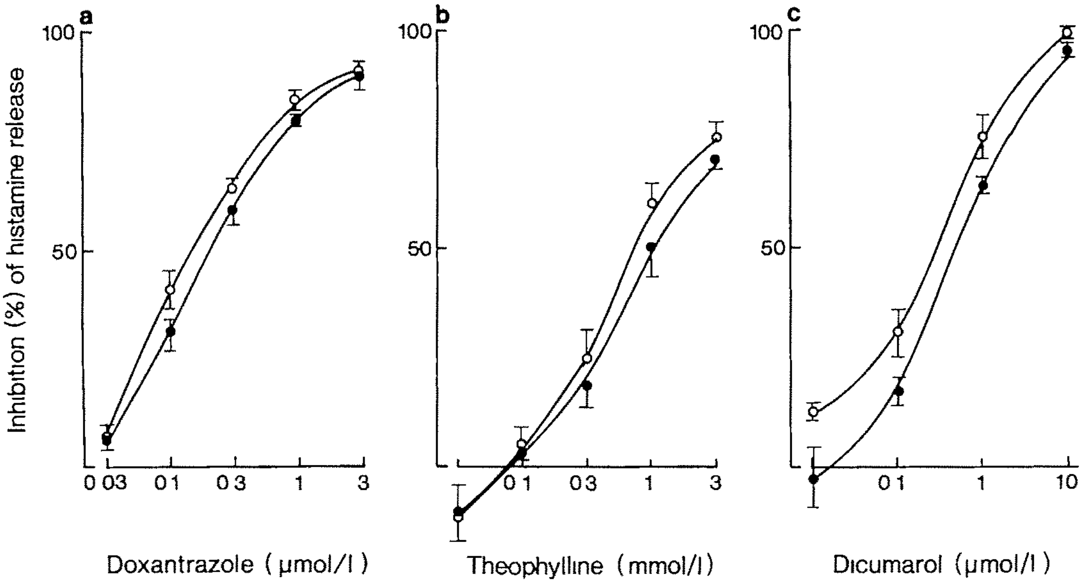

Figure 1 Inhibition (%) of histamine release from rat peritoneal cells incubated at 37°C for 30 min in either glucose-free (●) or complete (○) Tyrode solutions before the addition of a solution containing dextran, phosphatidyl serine and where appropriate various concentrations of inhibitor which was in (a) doxantrazole, (b) theophylline and (c) dicumarol. Each point is the mean of three separate experiments. Vertical lines show s.e. mean. Inhibition in the presence and absence of glucose was not significantly different at the 5% level when compared by Student's *t* test

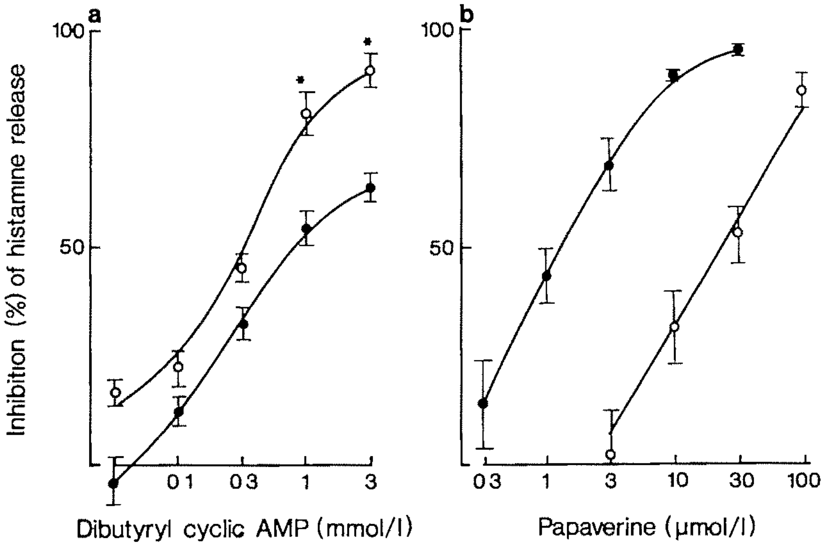

Figure 2 Inhibition (%) of histamine release from rat peritoneal cells incubated at 37°C for 30 min in either glucose-free (●) or complete (○) Tyrode solution before the addition of a solution containing dextran, phosphatidyl serine and where appropriate various concentrations of inhibitor which was in (a) dibutyryl cyclic AMP and in (b) papaverine. Each point is the mean of three separate experiments, vertical lines show s.e. mean. * $P < 0.005$ (by *t* test).

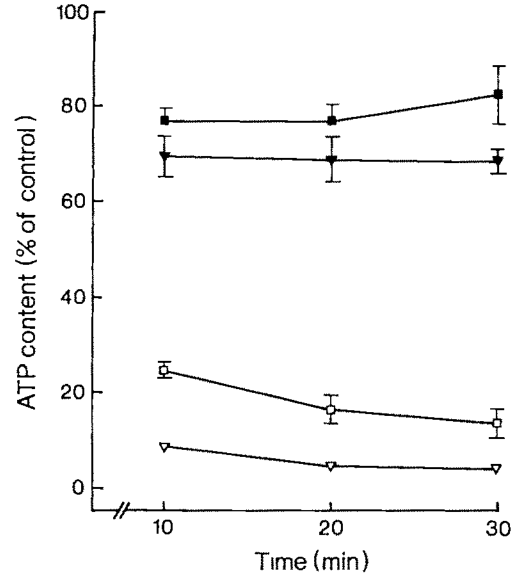

Figure 3 The ATP content of mast cells incubated for 10 to 30 min at 37°C in either complete (■,▼) or glucose-free (□, ▽) Krebs-Ringer soluton containing either papaverine 3 μmol/l (squares) or antimycin A 1 μmol/l (triangles). Each point is the mean of three separate experiments; vertical lines show s.d.

contrast to each of the foregoing inhibitors, papaverine was 20 times more active when added with the histamine-releasing stimulus to cells preincubated in glucose-free Tyrode solution than it was when added to cells in complete Tyrode (Figure 2b).

In order to examine further the effect of these compounds on mast cell energy metabolism, direct measurements were made of the cellular ATP content. As shown in Table 1, incubation of purified mast cells in glucose-free medium for 10–30 min at 37°C with doxantrazole (3 μmol/l), theophylline (3 mmol/l) or dibutyryl cyclic AMP (3 mmol/l) did not alter their ATP content. Furthermore, dicumarol did not reduce the cellular ATP content until concentrations of 56 or 100 μmol/l were used (Table 2). The fall in ATP content produced by dicumarol (100 μmol/l) was less in cells incubated in Krebs-Ringer medium containing glucose (Table 2).

When mast cells were incubated with papaverine (3 μmol/l) in the absence of glucose their ATP content fell by 75% below the control value after 10 min and became further reduced when incubation was continued for up to 30 min (Figure 3). However, in the presence of glucose, the reduction of ATP levels by papaverine was only about 25%. This effect of papaverine was compared with that of antimycin A. As shown in Figure 3, antimycin A (1 μmol/l) reduced the ATP content almost completely, this reduction also being largely counteracted when the mast cells were incubated in glucose-containing medium.

Discussion

Histamine release from rat mast cells incubated in glucose-free medium is more susceptible to inhibitors of oxidative metabolism, such as antimycin A, than release from cells in glucose-containing medium (Peterson, 1974; Diamant *et al.*, 1974). This is, presumably, because in the absence of glucose, mast cells are unable to derive sufficient ATP from glycolysis to support histamine release. Figures 1a and 1b show that both doxantrazole and theophylline inhibited histamine release from cells incubated in glucose-free medium to the same extent as from cells in complete medium. Furthermore, neither compound reduced the ATP content of mast cells (Table 1). Therefore, it is unlikely that these two compounds inhibit histamine release through an effect on energy metabolism, a conclusion that is consistent with previous observations (Garland & Mongar, 1976).

When incubated with rat mast cells, dicumarol reduced their ATP content apparently through inhibition of oxidative metabolism since the effect was reversed by glucose (Table 2). However, the concentration required to reduce the ATP level was higher

Table 1 The ATP content, expressed as a percentage of control, of mast cells incubated at 37°C in glucose-free medium for various periods with dibutyryl cyclic 3',5'-adenosine monophosphate (3 mmol/l), theophylline (3 mmol/l), or doxantrazole (3 μmol/l).

Incubation (min)	*ATP content*		
	Dibutyryl cyclic AMP	*Theophylline*	*Doxantrazole*
10	99.9±3.7	101.2±0.6	101.8±1.7
20	97.9±2.5	102.4±0.5	102.4±4.4
30	99.2±3.6	106.2±5.6	104.3±2.4

Values are mean ± s.e. mean of 3 separate experiments.

Table 2 The ATP content, expressed as a percentage of control of mast cells incubated at 37°C in either glucose-free or complete (*) medium for 30 min with various concentrations of dicumarol.

Dicumarol (μmol/l)	*ATP content*
10	93.9 ± 3.8 (9)
17.8	94.6 ± 0.2 (3)
31.6	92.8 ± 3.2 (3)
56.2	6.7 ± 1.9 (3)
100	3.9 ± 0.2 (3)
100 (*)	55.6 ± 2.1 (3)

Values are mean ± s.e. mean. Figures in parentheses indicate number of experiments.

than that which, in separate experiments, completely inhibited the dextran-induced release of histamine. Most notably, there was no reduction of ATP content by dicumarol after 30 min incubation at a concentration of 32 μmol/l, while a concentration of 10 μmol/l added concomitantly with the dextran and PS completely inhibited histamine release. Consistent with these findings, dicumarol inhibited equally histamine release from cells preincubated in either glucose-free or glucose-containing Tyrode (Figure 1c). These two pieces of evidence support the conclusion from earlier experiments (Garland & Mongar, 1976) that dicumarol prevents histamine release by a mechanism separate from its effect on energy metabolism.

Papaverine was a more potent inhibitor of histamine release from cells preincubated in glucose-free Tyrode than from cells in complete Tyrode (Figure 2b). Furthermore, direct measurements of the mast cell ATP content show a similarity of action between papaverine (3 μmol/l) and antimycin A (1 μmol/litre). Both compounds reduced the cellular ATP content, the reduction being greater in cells incubated in glucose-free medium. These findings support the hypothesis that papaverine inhibits dextran-induced histamine release from rat mast cells through an effect on oxidative metabolism (Garland & Mongar, 1976) even when the inhibitor is added at the same times as the releaser. In agreement with these observations, Fredholm, Guschin, Elwin, Schwab & Uvnäs (1976) found that inhibition of Compound 48/80-induced histamine release by a high concentration of papaverine (100 μmol/l) was accompanied by depletion of mast cell ATP content.

Inhibition of release by dibutyryl cyclic AMP was significantly greater when cells were preincubated in complete rather than in glucose-free Tyrode solution. This suggests that dibutyryl cyclic AMP requires glucose for its action, possibly associated with its transport into the cell. This hypothesis is consistent with the gradual development of inhibition observed when cells are incubated with dibutyryl cyclic AMP before the addition of the histamine-releasing agent (Stechschulte & Austen, 1973; Garland, 1975). However, further experiments are required to clarify this suggestion.

The technical assistance of Miss Susanne Kristensen, Mrs Annette Rasmussen, Mr J.J. Adcock and Mr H.C. Cox is gratefully acknowledged.

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(Received February 1, 1977.
Revised April 6, 1977.)

Br. J. Pharmac. (1977), **61**, 243–250

THE EFFECTS OF ALLOXANATE, NICOTINIC ACID AND IMIDAZOLE ON SECRETORY PROCESSES AND THE ACTIVITIES OF ADENYLATE CYCLASE AND 3′,5′-AMP PHOSPHODIESTERASE IN CAT PANCREAS

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1 Nicotinic acid and alloxanate inhibited water and electrolyte secretion in a dose-dependent fashion when added to the perfusate of the isolated saline-perfused pancreas of the cat stimulated by a supramaximal dose of secretin.

2 There were no changes in the concentration of sodium or potassium secreted into the juice, but the anions exhibited changes which were related to flow rate. As the flow rate declined the chloride concentration increased with a reciprocal decrease in bicarbonate concentration.

3 Nicotinic acid and alloxanate inhibited enzyme secretion stimulated by carbachol.

4 Imidazole inhibited pancreatic electrolyte secretion, but stimulated amylase secretion. Atropine (0.14 μM) reduced the secretion of amylase but did not abolish the effect.

5 Adenylate cyclase prepared from cat pancreas, was stimulated by the octapeptide of cholecystokinin-pancreozymin, secretin and sodium fluoride.

6 Alloxanate strongly inhibited both basal and hormone-stimulated adenylate cyclase activity. Nicotinic acid and imidazole stimulated basal adenylate cyclase activity but had little effect on secretin-stimulated activity.

7 Alloxanate, nicotinic acid and imidazole were all without effect on phosphodiesterase when tested in the presence of micromolar concentrations of adenosine 3′,5′-monophosphate (cyclic AMP). At higher cyclic AMP concentrations (2 mM) alloxanate and nicotinic acid were without effect, whereas imidazole had a slight stimulatory effect at 10 mM which was more marked at 50 mM.

8 Alloxanate (10 mM) strongly inhibited both basal and secretin-stimulated adenylate cyclase activity.

9 It is concluded that the effects of nicotinic acid, alloxanate and imidazole on pancreatic secretion are not mediated entirely through their effects on the adenylate cyclase or phosphodiesterase enzyme systems.

Introduction

The exocrine pancreas is responsible for two major secretory processes, electrolyte secretion and enzyme secretion. Electrolyte secretion is the transport of ions across the cell membrane which, due to the osmotic gradient created, is responsible for water movement in isotonic proportions. In the case of the pancreas, the dominant ions of the primary secretion are Na^+ and HCO_3^-, though the exact nature of the ion transport processes involved remains uncertain. Enzyme secretion is the discharge of digestive hydrolases from pre-formed zymogen granules following fusion of the granule membrane with the cell membrane in the process of exocytosis. There is some evidence also for an extragranular route of enzyme secretion. Enzyme secretion is a function of the acinar cells, which comprise 89% of the gland volume (Bolender, 1974). Most current evidence suggests that electrolyte secretion is a function of ductular elements within the gland, which comprise less than 4% of the total gland volume (Bolender, 1974). The chief stimuli to secretion are the gastrointestinal hormones secretin and cholecystokinin-pancreozymin (CCK-Pz) and the vagus nerve, via its transmitter substance acetylcholine, although other gastrointestinal hormones (e.g. vasoactive intestinal peptide and gastrin) can also stimulate secretion. Although there is some species variation, generally speaking secretin (and vasoactive intestinal peptide) stimulate largely electrolyte secretion, whereas CCK-Pz and acetylcholine (and gastrin) evoke largely enzyme secretion.

A role for adenosine 3',5'-monophosphate (cyclic AMP) has been claimed in both secretory processes. However, because of the heterogeneity of the gland and because all, or most, stimuli have multiple sites of action, it has not yet been possible for either secretory process to satisfy absolutely those criteria regarded by Sutherland as the minimum requirement for implicating cyclic AMP in the response to a given stimulus. One approach to this problem is to test the action of agents known to alter the activity of adenylate cyclase or 3',5'-AMP phosphodiesterase. Cholera toxin is one agent which is thought specifically to activate adenylate cyclase; its actions on the pancreas have been recorded elsewhere (Kempen, de Pont & Bonting, 1975; Smith & Case, 1975). In this paper we correlate the actions of some other agents (alloxanate, nicotinic acid and imidazole) on secretory processes in the isolated pancreas of the cat with their effects on adenylate cyclase and phosphodiesterase activity in cat pancreas homogenates. Alloxan has been reported to inhibit adenylate cyclase of the β-cell of the Islet of Langerhans (Cohen & Bitensky, 1969); nicotinic acid has been shown to lower the concentration of cyclic AMP in adipose tissue (Butcher, Baird & Sutherland, 1968); high concentrations of imidazole have been reported to stimulate mammalian phosphodiesterase (Butcher & Sutherland, 1962). The cat pancreas is a good model of pancreatic secretory processes because there is no basal electrolyte secretion and also because the action of a given stimulus is more specific for a given secretory process than in some other species. The properties of cat pancreatic adenylate cyclase will be described elsewhere (Kempen, de Pont & Bonting, unpublished). Preliminary accounts of some of the work described here have been published (Porter & Scratcherd, 1973; Bonting, Case, Kempen, de Pont & Scratcherd, 1974; Scratcherd, 1974).

Methods

Experiments on perfused cat pancreas

Experiments were performed on a saline-perfused preparation of the cat's pancreas prepared, with slight modifications, as described in detail elsewhere (Case, Harper & Scratcherd, 1968). Briefly, the pancreas was surgically isolated from cats of either sex weighing 1–3.4 kg, which had been denied food 18 h before the experiment. Perfusion fluid was led from a reservoir, through a heat-exchange coil (maintained at 38°C) and, by means of a roller pump, infused into the arterial supply of the gland (either the coeliac and superior mesenteric arteries via a cannula in the aorta, or in some experiments the coeliac artery alone). The effluent from the gland was drained from the superior mesenteric vein after occlusion of the portal tract. The standard perfusion fluid, isosmolal with cat plasma, had the following composition (mM): NaCl 125, KCl 4.3, $NaHCO_3$ 25, $MgSO_4$ 1.0, NaH_2PO_4 1.0, $CaCl_2$ 2.5 and glucose 5. Unless otherwise stated, drugs (nicotinic acid, alloxan (BDH, Chemicals Ltd.) and imidazole (Sigma Chemicals Ltd.)) were added to this perfusion fluid at concentrations of 3, 8 and 13 mM without compensatory reduction in NaCl concentration since osmotic changes of this magnitude have relatively little effect on secretory rate from this preparation (Case *et al.*, 1968). Perfusion fluids were filtered before use and gassed continuously with a mixture of 95% O_2 and 5% CO_2 to maintain pH at 7.4. A bank of four reservoirs allowed rapid changes in the composition of the perfusion fluid to be made. Throughout all experiments pancreatic electrolyte secretion was stimulated maximally by infusing secretin (prepared by the method of Crick, Harper & Raper, 1949) into the arterial cannula by means of a motor-driven syringe. Enzyme secretion was evoked by injecting either acetylcholine, carbachol (Sigma) or CCK-Pz (GIH Laboratory, Stockholm, Sweden) in a small volume of 0.9% w/v NaCl solution (saline, 0.5–1.0 ml) over 10 seconds. Pancreatic juice samples were collected in weighed plastic tubes. Bicarbonate concentrations were measured immediately after collection with a Natelson microgasometer; sodium and potassium concentrations were measured by flame photometry (Mark II, Evans Electroselenium Ltd., or Corning-EEL Model 430); chloride was measured potentiometrically (Model 92 chloridometer, Evans Electroselenium Ltd. or Buchler Digital chloridometer). Where necessary, perfusate osmolalities were checked with conventional osmometers (Osmet Precision Osmometer, Precision Systems Ltd., or Kanuer osmometer, Herber Knauer & Co.). As an index of total enzyme secretion, amylase activity was determined by digestion of starch at 30°C for 15 min using either the method of Nørby (Lagerlöf, 1942) or the method of Bernfield (1955); amylase activity is expressed as international units (iu).

Activity of adenylate cyclase and phosphodiesterase in cat pancreas

Cats of either sex, weighing 2–3 kg and fed *ad libitum*, were killed by rapid intracardial injection of pentobarbitone (90 mg/kg). The pancreas was removed, freed from adhering fat, minced with scissors and homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 15 ml of a solution containing: 10 mM Tris-HCl buffer, 2.5 mM MgCl$_2$, 2.5 mM disodium edetate (Na$_2$ EDTA) and 0.2 mg/ml soybean trypsin inhibitor. For adenylate cyclase experiments the buffer was adjusted to pH 7.4 and for phosphodiesterase experiments to pH 8.5. In the latter case the homogenate was stored without further treatment in 1 ml aliquots at −70°C to await assay. For adenylate cyclase studies the homogenate was centrifuged at 8000 *g* for 5 min in the cold, the pellet washed twice in the same homogenization medium and finally resuspended in 5 ml. The particulate suspension was divided into 0.5 ml aliquots and stored at −70°C to await assay. No decrease in enzyme activities was detected after 3 months storage. Adenylate cyclase activity was determined according to Rutten, de Pont & Bonting (1972). The assay medium had the following final composition (pH 7.4): α-[^{32}P]-adenosine triphosphate 0.4–0.6 mM, Tris-HCl 45 mM, MgCl$_2$ 5.5 mM, theophylline 10 mM, phosphoenolpyruvate 10 mM, pyruvate kinase 0.2 mg/ml, Na$_2$EDTA 0.5 mM and soybean trypsin inhibitor 40 μg/ml. The adenylate cyclase was tested with or without the following substances: secretin (donated by Dr M. Wünsch, Max Planck Institute for Biochemistry, Munich, G.F.R.), the C-terminal octapeptide of cholecystokinin-pancreozymin (PzO, a gift of Dr M. Ondetti, The Squibb Institute for Medical Research, Princeton, N.J., U.S.A.) and sodium fluoride. When hormone effects were studied, 1 mg/ml phosphatidylserine was also included in the assay medium to maintain a constant rate of cyclic AMP formation during the 10 min incubation at 37°C (Kempen, DePont & Bonting, 1974).

Phosphodiesterase activity was determined by the method of Rutten, Schoot & de Pont (1973), using substrate ([^{3}H]-cyclic AMP) concentrations of 1 or 4 μM for the low K_m activity and 2 mM for the high K_m activity. The other assay constitutents were: Tris-HCl (pH 8.5) 66 mM, MgCl$_2$ 1.0 mM and soybean trypsin inhibitor 40 μg/ml. The amount of substrate conversion was kept below 30%. Incubations were for 15 min at 37°C.

Results

Effects on the perfused cat pancreas

Nicotinic acid and alloxan. When these two drugs were added to the perfusate the solution becomes more acid. The pH was therefore adjusted to 7.4 at 37°C by addition of sodium hydroxide. Alloxan is a very unstable compound and in neutral solution is rapidly converted into alloxanate. The half life of alloxan at pH 7.4 and 37°C is about 1 min (Webb, 1966). Therefore, effects of addition of alloxan are probably due to alloxanate. Exposure of the pancreas to nicotinic acid or alloxanate at neutral pH caused a dose-dependent inhibition of secretion. Whereas both drugs had identical effects at 3 mM, nicotinic acid was more effective at concentrations of 8 and 13 mM (Figure 1). The responses were qualitatively similar at all concentrations tested, so only the electrolyte changes at 13 mM will be documented. The output of pancreatic juice and the concentration of electrolytes secreted in the third 10 min test period during perfusion with nicotinic acid or alloaxan were compared with the control period immediately before the addition of the drugs. In the case of alloxanate the volume of secretion fell by a mean of 0.28 ± 0.015 g/min ($n=6$) (a fall to 63.3% of the maximal rate), but the concentrations of the monovalent cations were almost unchanged. The concentration of sodium was 163.3 ± 1.26 mM ($n=6$) in the control period and 160.8 ± 0.75 mM ($n=6$) in the test period. The corresponding figures for potassium were 5.18 ± 0.03 mM ($n=6$) and 4.95 ± 0.02 mM ($n=6$) respectively. Nicotinic acid reduced secretion rate by a mean of 0.43 ± 0.045 g/10 min (a fall to 47% of the maximal rate) and as in the case of alloxanate, the monovalent cation concentrations of sodium in control and test period were 161.7 ± 0.5 mM ($n=6$) and 161.8 ± 1.3 ($n=6$) respecitvely and the corresponding figures for potassium were 4.9 ± 0.10 mM ($n=6$) and 5.13 ± 0.06 mM ($n=6$). The changes in anion concentration were very variable, the absolute values depending upon the rate of secretion. Inhibition with either alloxanate or nicotinic acid caused the

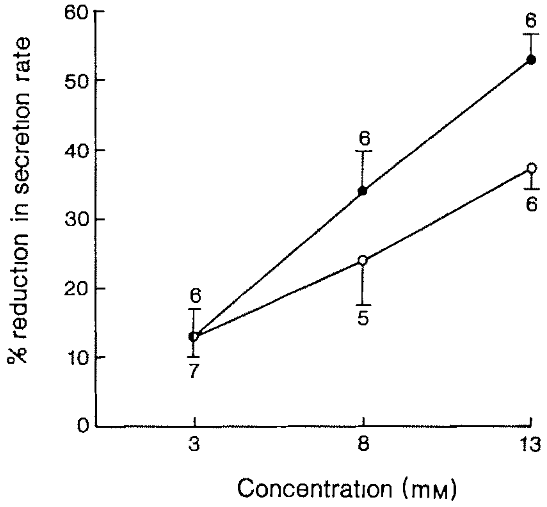

Figure 1 The percentage by which the maximum secretion rate was reduced by nicotinic acid (●) and alloxan (○), at the concentration stated. Bars indicate s.e. mean; no. of experiments beside each point.

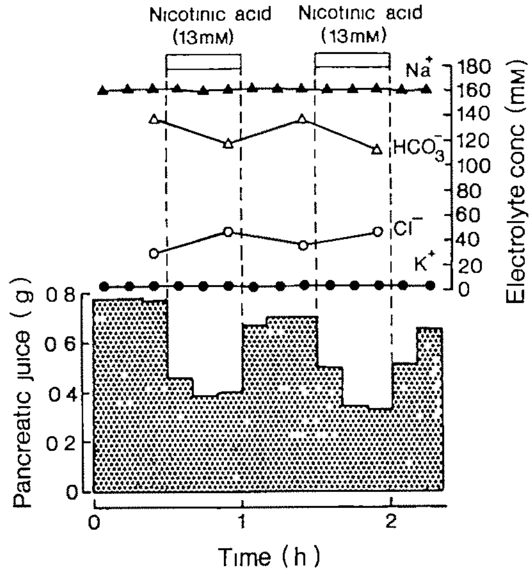

Figure 2 The effect of nicotinic acid 13 mM on the volume and electrolyte secretion of the maximally stimulated perfused pancreas.

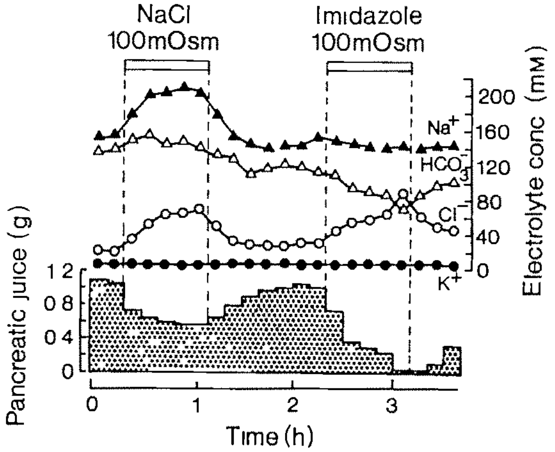

Figure 3 Comparison of equiosmolar solutions of sodium chloride and imidazole on the volume and electrolyte secretion in the perfused pancreas. Note the absence of an effect on the sodium concentration of the juice when imidazole 100 mM was added to the perfusate.

bicarbonate concentration to fall, accompanied by a reciprocal rise in chloride concentration (Figure 2). However, these changes were not different from those caused by reducing secretion rate to similar values by varying the dose of secretin.

The amylase secretion in response to a standard dose of 100 ng carbachol given during the third 10 min test period was expressed as a percentage of the mean response before the test period. A dose of 100 ng of carbachol was chosen because it evokes a consistent amylase secretion from the gland; with larger doses the response declines with repeated stimulation.

An inhibition of enzyme secretion occurred so that at 13 mM the response was reduced by 36 ± 6% ($n = 5$) in the presence of nicotinic acid and by 54 ± 11% ($n = 5$) in the presence of alloxanate.

Imidazole. In order to stimulate purified beef heart phosphodiesterase with imidazole, Nair (1966) had to use concentrations of the order of 10–100 mM. Since addition of imidazole to the perfusate at such concentrations would be likely to cause inhibition by an osmotic effect (Case *et al.*, 1968), in 4 experiments the effects of 100 mM imidazole addition were compared with the effects of adding an osmotically equivalent amount (50 mM) of sodium chloride. All experiments gave similar results; one is illustrated in Figure 3. The inhibition due to imidazole was greater than that produced by sodium chloride. More significantly, unlike the inhibitory effects of alloxanate and nicotinic acid, that due to imidazole was largely irreversible even after prolonged return to perfusion with normal fluid (2.5 h being the longest time tested).

The lack of any increase in ($Na^+ + K^+$) and ($HCO_3^- + Cl^-$) concentrations during exposure to 100 mM imidazole suggests that imidazole was appearing in the pancreatic juice at approximately the same concentration as in the perfusate.

Because of the lasting inhibition of electrolyte secretion by high concentrations of imidazole (which effectively precluded observations on enzyme secretion), concentrations of 20 mM or less were tested in six other experiments. At such concentrations, imidazole was usually without effect on electrolyte secretion. However, basal enzyme secretion was stimulated; and stimulation was detectable at concentrations as low as 2.5 mM imidazole. This stimulation of enzyme secretion was largely, but not entirely, abolished by atropine at concentrations (0.14 μM) known to block the effect of acetylcholine released endogenously from nerve terminals within the gland by excess potassium (Argent, Case & Scratcherd, 1971) (Figure 4).

Effects on cat pancreas adenylate cyclase and phosphodiesterase activities

Adenylate cyclase is stimulated by the addition of pancreozymin-c-octapeptide, secretin and sodium fluoride (Table 1).

The effects of alloxan on adenylate cyclase were tested under two conditions. First, to avoid alloxonate formation, alloxan was dissolved in ice-cold twice

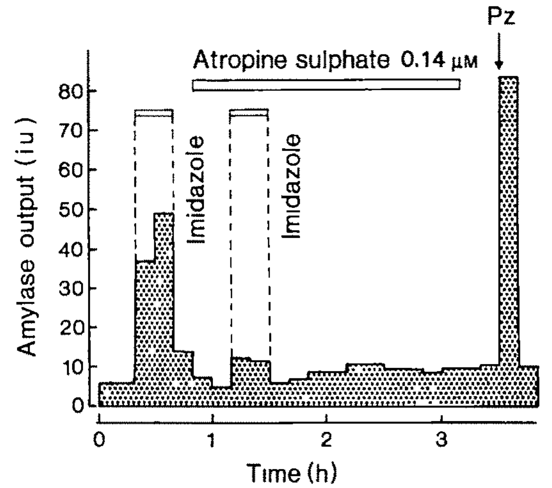

Figure 4 The effect of imidazole on amylase secretion by the pancreas. For the duration of open bars imidazole 20 mM was added to the perfusate, before and after the addition of atropine sulphate (14 µM). At Pz, 0.15 mg of Crick, Harper & Raper (1949) pancreozymin was injected into the arterial line of the perfusion apparatus to demonstrate that the pancreas was capable of responding to an enzyme stimulant. Fluid secretion maximally stimulated throughout with secretin.

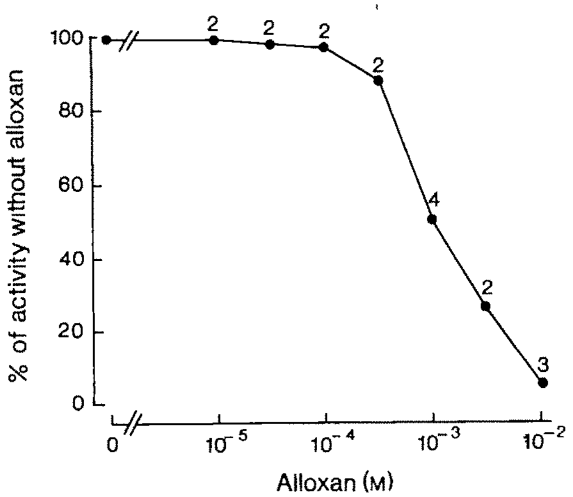

Figure 5 Effect of increasing concentrations of alloxan on cat pancreas adenylate cyclase activity stimulated by secretin 1 µM. Activities are expressed as the percentage of the activity obtained in the absence of alloxan in the same experiment and are given as means; for each concentration the number of experiments is shown. Phosphatidylserine (1 mg/ml) is present in all assays.

distilled water with adjustment to pH 7.4 as late as possible before its addition to the adenylate cyclase medium and the start of incubation. Secondly, to promote alloxanate formation, alloxan was preincubated for 15 min at 37°C before addition to the cyclase medium. Under both conditions exactly the same inhibition of adenylate cyclase activity was observed, which presumably is therefore an effect of alloxanate. At 10 mM alloxanate strongly inhibited basal and secretin-stimulated activities (Table 2). Inhibition of pancreozymin- and NaF-stimulated activity was also observed. The concentration of alloxanate required for 50% inhibition of the secretin-stimulated activity was about 1 mM (Figure 5). Addition of 10 mM cysteine to the incubation buffer completely prevented the inhibitory action of alloxanate on adenylate cyclase activity. Both nicotinic acid and imidazole (10 mM) stimulated basal adenylate cyclase activity, but had little effect on secretin-stimulated activity (Table 2). At 50 mM the stimulatory effect of imidazole was more pronounced (Table 2). When these substances were added to the isolated pancreas in the absence of secretin, there was no stimulation of pancreatic secretion.

Table 1 Properties of cat pancreas adenylate cyclase

Stimulus	*Activity (pmol cyclic AMP formed 10 min⁻¹ mg prot⁻¹)*
	112 ± 14 (32)
PzO (1 µM)	208 ± 14 (27)
Secretin (1 µM)	396 ± 25 (23)
NaF (10 mM)	655 ± 33 (11)

Values represent means of activities in a 6 min 8000 *g* particulate fraction with s.e.; the number of experiments is given in parentheses. PzO = the C-terminal octapeptide of cholecystokinin-pancreozymin.

Phosphodiesterase. The effects of all three drugs on cat pancreatic phosphodiesterase activity are presented in Table 3. Alloxanate (10 mM), nicotinic acid (10 mM) and imidazole (10 mM and 50 mM) were all without effect when tested in the presence of micromolar concentrations of cyclic AMP. At higher cyclic AMP concentration (2 mM) alloxanate and nicotinic acid were again without effect, whereas imidazole stimulated activity slightly at 10 mM and more markedly at 50 mM.

Discussion

The results of this study are unfortunately not as clear-cut as we had anticipated when embarking upon the project. The actions of alloxan, nicotinic acid and imidazole on cat pancreatic adenylate cyclase and phosphodiesterase are not consistently correlated with an action on pancreatic fluid or enzyme secretion. Alloxan, acting probably as alloxanate, and nicotinic acid, both inhibited fluid secretion and enzyme secretion by the perfused pancreas of the cat. During perfusion with either drug there were no changes in the concentrations of sodium or potassium. However, the osmolality of perfusate and juice were equal. The fall in bicarbonate concentration and rise in chloride concentration produced by both drugs simply reflect the redution in secretory rate which in this and other pancreas preparations is known to produce these changes (Case *et al.*, 1968). The gradual decline in bicarbonate concentration with time is a feature of the saline-perfused cat pancreas and has been discussed previously (Case *et al.*, 1968). Alloxan has long been known to cause profound vacuolation of pancreatic ductal epithelium after a single intravenous dose of 50 mg/kg (Goldner & Gomori, 1943), and this necrosis is responsible for a reduced electrolyte secretion by the gland (Grossman & Ivy, 1946; Tiscornia, Janowitz & Drieling, 1968). It seemed to have no permanent deleterious effect on the isolated gland over the time tested in these experiments. No effects of nicotinic acid on pancreatic function have previously been published, though it apparently causes stimulation of gastric acid secretion in humans and dogs with Pavlov pouches (Andersson, Carlson, Orö & Richards, 1971). Alloxanate has a strong inhibitory effect on basal and stimulated pancreatic adenylate cyclase activity. Alloxan is an unstable SH reagent and its effects on adenylate cyclase may depend upon inactivation of SH groups in the cyclase molecule since inhibition could be prevented by cysteine. The inhibitory effect of alloxanate on pancreatic secretion may depend on such inhibition, or by inactivation of SH groups on the serosal membrane of pancreatic cells known to be involved in the action of secretin (Wizeman, Schulz & Simon, 1973).

Nicotinic acid has been claimed to inhibit fat cell adenylate cyclase (Skidmore, Schoenhoefer & Kritchevsky, 1971). It has also been reported to stimulate fat cell phosphodiesterase (Krishna, Weiss, Davies & Hynie, 1966; Schwabe & Ebert, 1969; Nakano, 1970) although the effects were small and have not been confirmed by others (Petersen, Hillman & Ashmore, 1968; Kupieki & Marshall, 1968; Therriault & Winters, 1970; Skidmore *et al.*, 1971; Mengel, Meyer, Ebert & Schwabe, 1972). Nicotinic

Table 2 *Effects of alloxanate, nicotinate and imidazole on basal and secretin-stimulated cat pancreas adenylate cyclase activities

	Basal	*Secretin (1 μM)*
Control	102 ± 15 (8)	366 ± 40 (8)
Control	100%	100%
Alloxanate (10 mM)	14 ± 5.8 (3)	5 ± 1.2 (4)
Nicotinate (10 mM)	179 ± 12 (3)	96 ± 8 (3)
Imidazole (10 mM)	280 ± 40 (4)	106 ± 6 (4)
Imidazole (50 mM)	428 (1)	133 (1)

Control activities are given in the first row as pmol cyclic AMP formed per 10 min per mg protein, and represent means ± s.e. of 8 different cat pancreas preparations.

*For each experiment activities in the presence of these drugs are expressed as a percentage of the control activity. The data in the lower part of the Table represent means ± s.e. of these percentage values, with the number of experiments (each with a separate cat pancreas preparation) given in parentheses.

Table 3 Effects* of alloxanate, nicotinate and imidazole on cat pancreas cyclic AMP phosphodiesterase activities at various substrate concentrations

	1 μM cyclic AMP	*4 μM cyclic AMP*	*2 mM cyclic AMP*
Control	2.4 ± 0.4 (4)	7.4 ± 0.9 (4)	41 ± 6.5 (4)
Control	100%	100%	100%
Alloxanate (10 mM)	108 ± 11 (3)	104 ± 17 (4)	104 ± 6 (4)
Nicotinate (10 mM)	89 ± 18 (3)	101 ± 10 (4)	103 ± 4 (4)
Imidazole (10 mM)	107 ± 4 (4)	100 ± 8 (4)	119 ± 6 (4)
Imidazole (50 mM)	97 ± 9 (4)	101 ± 5 (4)	157 ± 12 (4)

Control activities are given in the first row as nmol cyclic AMP hydrolysed per 15 min per mg protein.

* See legend to Table 2.

acid seemed to have little or no inhibitory effect on either adenylate cyclase or phosphodiesterase from cat pancreas; in fact it seemed to stimulate basal adenylate cyclase activity slightly. It is therefore unlikely that this drug causes inhibition of pancreatic electrolyte or enzyme secretion by lowering intracellular cyclic AMP concentration.

High concentrations of imidazole stimulate phosphodiesterase (Butcher & Sutherland, 1972) and it has been recently confirmed that it is only the high K_m enzyme which is involved (Donelly, 1976). It is likely that this enzyme is not of physiological significance at low cyclic AMP concentrations (Rutten, Schoot, de Pont & Bonting, 1973). Imidazole at a concentration of 50 mM stimulated pancreatic phosphodiesterase. However, the dominant effect was rather a stimulation of adenylate cyclase activity, which was less in the presence of secretin (Table 2). At the same time pancreatic electrolyte secretion was irreversibly inhibited and enzyme secretion stimulated. Enzyme secretion was largely due to release of acetylcholine from nerve terminals within the gland but partly due to a direct effect on the acinar cell. Both these effects (i.e. release of acetylcholine and pancreatic enzymes) are probably due to elevation of intracellular calcium concentration and not to activation of adenylate cyclase. Thus, in cardiac muscle, imidazole is able to evoke contractures (Miller & Chapman, 1972) by release of calcium from an intracellular store, probably the sarcoplasmic reticulum (Chapman, Rutherford & Wallace, 1976). In isolated pancreas of the rat, imidazole will increase calcium efflux from preloaded glands in the absence of extracellular calcium (R.M. Case, unpublished observations). The slight stimulatory effect of imidazole in the presence of atropine could be due to the release of calcium from some intracellular store, but the irreversible effect on electrolyte secretion remains unsolved.

In conclusion it would appear that the effects on pancreatic secretion of nicotinic acid, alloxanate and imidazole, cannot be mediated entirely through their effects on adenylate cyclase or phosphodiesterase.

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(Received February 1, 1977)

Br. J. Pharmac. (1977), **61**, 251–256

THE EFFECTS OF PENTOBARBITONE AND CHLORALOSE ANAESTHESIA ON THE VAGAL COMPONENT OF BRONCHOCONSTRICTION PRODUCED BY HISTAMINE AEROSOL IN THE ANAESTHETIZED DOG

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1 Total lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were measured in dogs anaesthetized with pentobarbitone or chloralose and subjected to aerosols of histamine during 4 successive inspirations.

2 Histamine caused concentration-dependent increases in R_L and decreases in C_{dyn}. A significant vagal component was involved, but only when chloralose was employed and then only in the R_L response.

3 The resting values of R_L and C_{dyn} were similar regardless of which anaesthetic was used and remained essentially the same if the vagi were cooled.

4 Electrical stimulation of the efferent vagi caused large increases in R_L of dogs given chloralose and these effects were attenuated by the administration of pentobarbitone. Such stimulation was relatively ineffective in dogs given pentobarbitone alone.

5 *In vitro*, electrical field stimulation caused contractions of dog trachealis muscle. The responses were reduced by pentobarbitone in concentrations approximating to plasma levels in the anaesthetized dogs (1 to 5×10^{-4} M), but the effects of exogenous acetylcholine were unaltered. The inhibition was dose-dependent, reversed by washing and unaltered by hexamethonium.

6 The results suggest that pentobarbitone inhibits the vagal component of histamine-induced bronchoconstriction in the dog by an action on the efferent pathway. Furthermore, pentobarbitone acts either by blocking transmission along postganglionic parasympathetic nerves or by preventing the release of acetylcholine from the nerve endings in the lung.

Introduction

Studies both in animals and man have shown that many different bronchoactive agents can cause a vagally mediated bronchoconstriction (Gold, 1973). Although the effects of local anaesthetic aerosols on reflex bronchoconstriction have been investigated in the dog (Dain, Boushey & Gold, 1974; 1975; Cross, Guz, Jain, Archer, Stevens & Reynolds, 1976) little is known about the effects of intravenous general anaesthetics on this mechanism of bronchoconstriction. For this reason we have investigated the effects of two intravenous general anaesthetics commonly used in animal experimentation, namely chloralose and pentobarbitone, on the reflex component of histamine-induced bronchoconstriction in dogs. We have also conducted *in vitro* experiments, with canine isolated trachealis muscle, to help explain our *in vivo* findings. Parts of this work have been communicated to the British Pharmacological Society (Richards & Jackson, 1976).

Methods

In vivo *experiments*

Light surgical anaesthesia was induced in Beagle dogs of either sex (9–12 kg) by the intravenous injection of pentobarbitone 30 mg/kg or chloralose 80 mg/kg. The level of anaesthesia was maintained by a continuous infusion of 0.1 mg kg^{-1} min^{-1} pentobarbitone or by a bolus injection of 10–15 mg/kg chloralose every 15 minutes. Immediately after induction of anaesthesia a cuffed Magill endotracheal tube was inserted, to be replaced after tracheotomy by a plastic cannula with its end just below the cricoid cartilage. The animals were ventilated with air at constant pressure (0.98 kPa) by means of a Bird Mark VII respirator. The lungs were inflated to 1.96 kPa transpulmonary pressure (TPP) after severe histamine-induced falls in lung compliance and also periodically to reverse the gradual decrease in lung volume, which occurs during positive pressure ventilation. A mid-line thoracotomy was carried out routinely.

A catheter was inserted into the muscularis branch of the right femoral artery for recording blood pressure, using a Statham P23Db pressure transducer and heart rate was derived from the blood pressure signal with a Devices instantaneous rate meter. Catheters were placed in the right medial saphenous vein and the muscularis branch of the left femoral artery for the injection of drugs and the removal of arterial blood for blood gas analysis respectively.

Both cervical vagosympathetic nerves were carefully exposed and placed on silver thermodes, 2.5 cm long, through which water at 0.5°C could be circulated. When the nerves were cooled, chilled saline (0.9% w/v NaCl solution) swabs at approximately 0.5°C were placed on the nerves which passed over the thermodes. In some dogs both nerves were placed on shielded bipolar platinum electrodes and stimulated by two Devices square wave stimulators with pulses of 1 ms width, supramaximal voltage, at a frequency of 20 Hz for 20 s periods.

The body temperature of the dogs was maintained at 38.5°C with a thermostatically controlled heating blanket in conjunction with a rectal thermocouple.

The partial pressures of oxygen ($P\text{O}_2$) and carbon dioxide ($P\text{CO}_2$) in the arterial blood and the blood pH were monitored regularly by means of a Radiometer ABL 1 acid-base analyser. By adjusting the ventilation of the animal at the beginning of the experiment, or during control periods, it was possible to maintain $P\text{O}_2 > 13.3$ kPa, $P\text{CO}_2$ between 3.32 kPa and 5.32 kPa and pH between 7.36 and 7.46.

Air flow rate was measured with a Fleisch pneumotachograph (Type 0; 9.8 Pa ≡ 43.16 ml/s) connected to a Furness Controls micromanometer (100–0–100 Pa). Tidal volume was obtained by electrical integration of the flow signal with a Devices integrator. TPP was measured by a Furness Controls micromanometer (1–0–1 kPa), one side of which was connected to the tracheal cannula and the other side of which was left open to the atmosphere. All recordings were displayed on a Devices M19 recorder.

Total lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were measured by a manual graphic method using the displayed signals of flow, volume and TPP (Amdur & Mead, 1958). The respiratory computer described by Carney, Pugh & Sheard (1972) was also used for the calculations of R_L and C_{dyn}, its displayed output being calibrated and checked for accuracy by comparison with simultaneous manual determinations of R_L and C_{dyn}. The computer was accurate to ± 0.02 kPa 1^{-1} s for R_L and to ± 6.2 ml kPa^{-1} for C_{dyn}.

Aerosols were generated on inspiration only using a Vaponefrin inhalajet nebuliser modified to deliver a significant proportion of large (12.8 µm) as well as small (0.5 µm) particles. The nebuliser was modified by removing the impingement ball.

The effects of histamine aerosols on resting R_L and C_{dyn} were investigated in 5 dogs anaesthetized with pentobarbitone (by the use of histamine solutions of 0.125, 0.5, 1.0 and 2.0%), or with chloralose (by the use of histamine solutions of 0.0625, 0.125, 0.25 or 0.5%). In addition, the effects of similarly administered aerosols of saline were investigated in two dogs anaesthetized with chloralose. The following experimental procedure was used: the vagus nerves were maintained at 38.5°C and 4 breaths were administered of histamine aerosol in a concentration selected at random from the previous list. Identical histamine challenges were repeated at 30 min intervals until two successive responses were consistent. Next the histamine was given after cooling both cervical vagi to approximately 0.5°C. The nerves were then rewarmed to 38.5°C and the procedure repeated with a different concentration of histamine. A total of 3 observations was made on the effects of vagal cooling on each concentration of histamine.

Measurement of plasma levels of pentobarbitone

Venous blood samples were taken from dogs approximately 4 h after the induction of pentobarbitone anaesthesia. After centrifugation, the plasma concentration of pentobarbitone was measured as described by Udenfriend, Duggan, Vasta & Brodie (1957).

In vitro *experiments*

Beagle dogs of either sex (9–12 kg) were anaesthetized with intravenous chloralose 80 mg/kg and the cervical trachea removed. Trachealis muscle preparations were then prepared from single tracheal rings (Yamaguchi, Hitzig & Coburn, 1976). The trachealis muscle was attached by thread to a tissue holder made from a 5 ml syringe case in the wall of which were fixed two platinum wires for electrical field stimulation of the muscle. The holder was placed in a 25 ml organ bath containing Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 and maintained at 38.5°C. Under a resting tension of 1 g, contractions of the muscle were measured isometrically with a Grass FT.03 transducer. At 10 min intervals, the tissue was stimulated for 15 s with square wave pulses (width 2 ms, frequency 20 Hz and supramaximal voltage) delivered from a Grass S4 stimulator and using a Devices Digitimer. When at least 4 consistent responses to field stimulation had been obtained pentobarbitone (1×10^{-4} M, 2.5×10^{-4} M or 5×10^{-4}M) was added to the organ bath and left until there was no further reduction in the response. The drug was then washed from the bath and the response recovered to control values. Atropine 1.8 µM was then added. Similar experiments were conducted in which hexamethonium 3.6 µM was added to the bath 30 min before the addition of pentobarbitone. In other

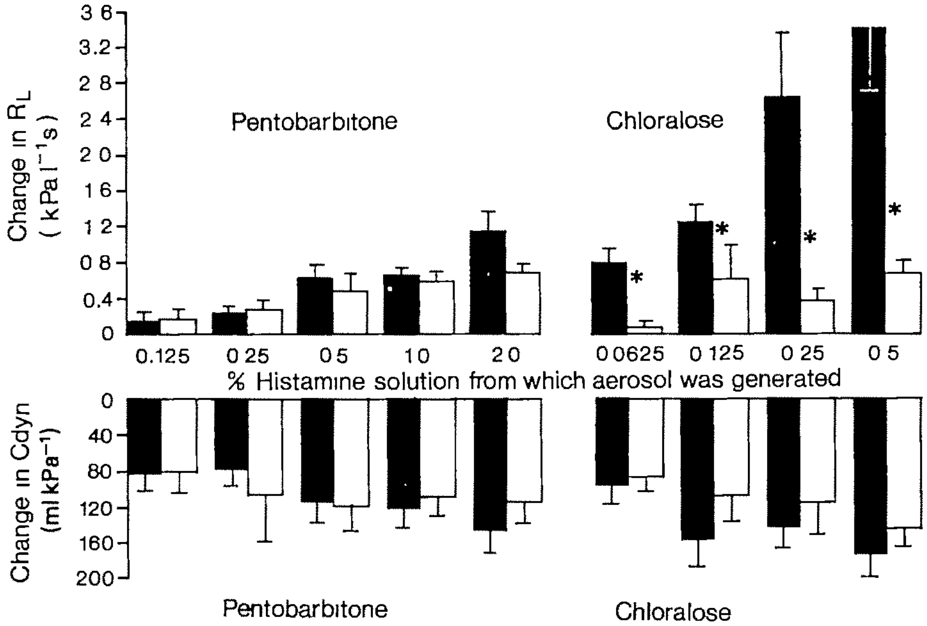

Figure 1 Changes in total lung resistance (R_L) and dynamic lung compliance (C_{dyn}) produced by 4 breaths of histamine aerosol generated from different concentrations of histamine solution in dogs anaesthetized with pentobarbitone or chloralose. The solid columns are the responses with the vagi at body temperature and the open columns are the responses with the vagi cooled to 0.5°C. Bars indicate s.e. mean, $n=6$ for solid columns, $n=3$ for open columns. * $0.05 > P > 0.01$.

experiments, cumulative dose-response curves to acetylcholine were obtained in the absence and presence of 5×10^{-4} M pentobarbitone. The pentobarbitone was added to the bathing solution and 30 min allowed before the first dose of acetylcholine was given.

Drugs and solutions

The drugs used were: histamine acid phosphate (BDH), chloralose (Koch-Light Laboratories), pentobarbitone sodium (Sagatal, May and Baker), acetylcholine chloride (Koch-Light Laboratories), atropine sulphate (Sigma) and hexamethonium bromide (May and Baker).

Drug solutions were freshly prepared in isotonic saline and concentrations are given in terms of bases.

The Krebs-Henseleit solution contained (g/l): NaCl 6.87, $MgSO_4 7H_2O$ 0.29, $NaH_2PO_4 2H_2O$ 0.18, dextrose 1.0, $NaHCO_3$ 2.1, KCl 0.43 and $CaCl_2$ 0.28.

Results

In vivo *experiments*

Four breaths of histamine aerosol administered to 5 dogs anaesthetized with pentobarbitone produced dose-dependent increases in R_L and falls in C_{dyn} (Figure 1). Cooling the cervical vagi did not significantly affect the increases in R_L or the decreases in C_{dyn} ($P > 0.05$).

In 5 dogs anaesthetized with chloralose, 4 breaths of histamine aerosol produced marked increases in R_L even when the aerosol was generated from comparatively low concentrations of histamine solution (Figure 1). The increases in R_L were significantly inhibited by vagal cooling, whereas the falls in C_{dyn} were unaffected ($P > 0.05$). Four breaths of an aerosol generated from normal saline did not affect R_L or C_{dyn}.

The initial resting values of R_L and C_{dyn} were similar regardless of the anaesthetic employed and cooling the cervical vagi did not significantly affect any of these values. The initial resting value of R_L in dogs anaesthetized with chloralose was 0.26 ± 0.05 kPa l^{-1} s compared with 0.23 ± 0.003 kPa l^{-1} s with pentobarbitone, whilst the respective values of C_{dyn} were 213 ± 25 ml kPa^{-1} and 245 ± 16 ml kPa^{-1}; all values are mean ± s.e. mean, $n=5$.

The results showed that histamine aerosol was unable to produce a large reflex bronchoconstriction in dogs anaesthetized with pentobarbitone. To establish whether this block was occurring on the afferent or efferent side of the reflex the vagus nerves were electrically stimulated. To prevent cephalad

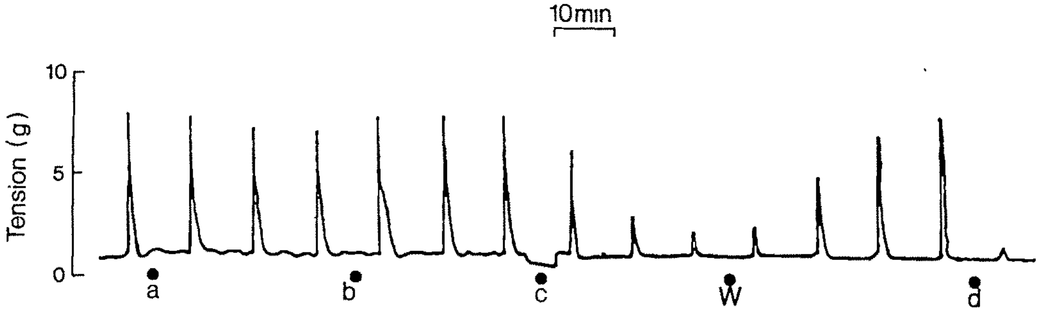

Figure 2 The effect of hexamethonium 1.8 μM (given at a and b); pentobarbitone 5×10^{-4} M (given at c) and atropine 1.8 μM (given at d) on the response of canine trachealis muscle to field stimulation. The bath was washed at W.

stimulation the electrodes were placed on the vagus nerves caudal to the point of cooling.

Stimulation of the vagi in dogs anaesthetized with pentobarbitone produced an increase in R_L of 0.12 ± 0.4 kPa l^{-1} s while in dogs anaesthetized with chloralose a much greater increase occurred in R_L of 1.2 ± 0.18 kPa l^{-1} s (mean ± s.e. mean, $n = 6$). In both groups of animals vagal stimulation stopped the heart with subsequent 'vagal escape'.

When intravenous pentobarbitone was given to dogs anaesthetized with chloralose the response to electrical stimulation of the vagus nerves 10 min later was significantly reduced although resting values of R_L were unaffected. Addition of 5 mg/kg reduced the response by 49% ± 14.5% and 10 mg/kg by 69.5% ± 11.3% (mean ± s.e. mean, $n = 7$).

Plasma levels of pentobarbitone

The plasma concentrations of the samples of venous blood taken from 4 dogs anaesthetized with pentobarbitone were 1.42×10^{-4} M; 1.73×10^{-4} M; 1.55×10^{-4} M and 1.73×10^{-4} M pentobarbitone. These values agree with those quoted by Altura & Altura (1975).

Table 1 The effect of acetylcholine on canine isolated trachealis muscle in the absence and presence of pentobarbitone 5×10^{-4} M

Acetylcholine (M)	*Response (g)*	*Response in presence of pentobarbitone (g)*
2.2×10^{-8}	0.4 ± 0.1	0.5 ± 0.2
8.8×10^{-8}	1.0 ± 0.3	1.1 ± 0.4
3.5×10^{-7}	2.1 ± 0.5	2.2 ± 0.6
1.4×10^{-6}	4.0 ± 0.7	4.2 ± 1.0
5.5×10^{-6}	5.4 ± 0.9	5.7 ± 1.2
2.2×10^{-5}	7.3 ± 1.2	7.6 ± 1.4
8.8×10^{-5}	9.5 ± 1.6	9.4 ± 1.6

In vitro *experiments*

Pentobarbitone 5×10^{-4} M did not affect the dose-response curve to acetylcholine (Table 1). However, 1×10^{-4}, 2.5×10^{-4} and 5×10^{-4} M pentobarbitone significantly inhibited the response of trachealis muscle to field stimulation by 29.4 ± 8.3% (mean ± s.e. mean, $n = 9$), 62.7 ± 7.2% ($n = 8$) and 64.4 ± 9.4% ($n = 7$) respectively. The maximum inhibition occurred 30 min after administration of pentobarbitone and the tissue had recovered completely 30 min after washing the drug from the bath. Hexamethonium 3.6 μM did not affect the response to field stimulation and in its presence pentobarbitone 5×10^{-4} M still inhibited the response (Figure 2). Atropine 1.8 μM blocked completely the effects of field stimulation in all the preparations used in this study.

Discussion

Dennis & Douglas (1970) showed that pentobarbitone protected guinea-pigs from the respiratory effects of histamine aerosol. Douglas, Dennis, Ridgway & Bouhuys (1972) suggested that this inhibition may have been caused by the action of pentobarbitone on the central nervous system resulting in a disturbance of the sympathetic and parasympathetic control of the airways. Our results extend these earlier observations.

In dogs anaesthetized with pentobarbitone we found it impossible to produce large reflex increases in R_L with histamine aerosol. In contrast, in dogs anaesthetized with chloralose, histamine aerosol produced a severe bronchoconstriction which was almost entirely reflex. Electrical stimulation of both cervical vagosympathetic nerves produced small increases in R_L (0.12 kPa l^{-1} s) with pentobarbitone anaesthesia and large increases (1.2 kPa l^{-1} s) with chloralose. Nerve stimulation stopped the heart in both groups of animals. Moreover, the addition of small quantities of pentobarbitone to dogs anaesthetized with chloralose significantly reduced the response of the airways to electrical stimulation of the cervical vagosympathetic nerves. We concluded, therefore, that pentobarbitone inhibited reflex bronchoconstriction by an action on the efferent arm of the reflex.

In dogs anaesthetized with chloralose the increase in R_L produced by electrical stimulation of the cervical vagosympathetic nerves was approximately 30% of the increase in R_L produced by 4 breaths of histamine aerosol generated from a 2.0% solution. The inability of electrical nerve stimulation to match the bronchoconstriction produced by reflex action can probably be attributed to the way in which the nerves were electrically stimulated in these experiments. Unlike the cat, the cervical vagus nerve of the dog cannot be separated from sympathetic fibres (Miller, 1964) unless the nerve is cut and a microdissection performed. In our experiments, where this was not done, sympathetic fibres were electrically stimulated with the cervical vagi. Olsen, Colebatch, Mebel, Nadel & Staub (1965) have shown, using the cat, that electrical stimulation of the cervical vagi is considerably more effective in increasing R_L than vagosympathetic stimulation.

In vitro experiments showed that pentobarbitone, at concentrations likely to be encountered in the plasma of dogs anaesthetized with this drug, did not affect the response of canine trachealis muscle to acetylcholine. Thus anaesthetic concentrations of pentobarbitone did not depress tracheal muscle directly or have an atropine-like action; however, they did significantly inhibit the response of trachealis muscle to field stimulation. The inhibition was concentration-dependent, reversible and occurred in the presence of a ganglion blocking agent. Since the response of the trachealis muscle to field stimulation was completely blocked by atropine we conclude that in this preparation pentobarbitone either prevents the release of acetylcholine from parasympathetic nerve endings, or inhibits transmission along postganglionic parasympathetic nerves. Further experiments are needed to establish which of these two proposed modes of action is operating. The barbiturates have been reported to have both local anaesthetic actions and an effect on transmission at autonomic neuroeffector junctions in a variety of tissues (Goodman & Gilman, 1975). The proposed modes of action for pentobarbitone in our experiments are therefore not novel for this class of drug.

It is interesting to note that the effects of vagal stimulation on the heart were not inhibited by pentobarbitone, indicating a selectivity of action of the drug on the parasympathetic post-ganglionic nerves in the lung.

Reflex bronchoconstriction produced by histamine aerosol is probably the result of an increase in the activity of lung irritant receptors which, via central connections, produces higher vagal tone to the airways (Mills, Sellick & Widdicombe, 1969; Gold, Kessler & Yu, 1972; Sampson & Vidruk, 1975). Sellick (1970) investigated the effect on lung irritant receptor activity in the rabbit of Nembutal (Abbott), a commercial preparation of pentobarbitone sodium dissolved in ethyl alcohol and propylene glycol. She found that Nembutal stimulated the irritant receptors and that this stimulation was caused by the solvent and not pentobarbitone, which did not affect the discharge pattern of the receptors. Sagatal, the preparation used in this study, is similar in constitution to Nembutal and therefore it is unlikely that it suppresses the activity of lung irritant receptors: more probably it stimulates them.

The role of the vagus nerves in histamine-induced bronchoconstriction in anaesthetized dogs is not clear. Gold *et al.* (1972) claim that histamine bronchoconstriction is entirely reflex, while others attribute the bronchoconstriction solely to the direct action of histamine on bronchial smooth muscle (Krell, Chakrin & Wardell, 1976). Our results may explain this particular discrepancy. Gold *et al.* (1972) used chloralose and urethane as the anaesthetic agents whereas Krell *et al.* (1976) used pentobarbitone. However, DeKock, Nadel, Zwi, Colebatch & Olsen (1966) using dogs anaesthetized with pentobarbitone, injected histamine directly into the bronchial arteries and obtained a reflex bronchoconstriction. The reason for the discrepancy between our and their observations may be related to the quantity of pentobarbitone administered because they used paralysed animals and did not indicate in their paper the maintenance dose of anaesthetic.

The changes in C_{dyn} produced by histamine aerosol were not significantly affected by cooling the vagus nerves in dogs anaesthetized with either pentobarbitone or chloralose. This finding is consistent with the view that the peripheral airways are not well innervated by the parasympathetic fibres of the autonomic nervous system (Nadel, 1974).

Although Fleisch & Calkins (1976) have pointed out some differences between the responses of rabbit tracheal and bronchial smooth muscle to certain pharmacological agents, the *in vitro* responses obtained in this study agree in most part with our *in vivo* observations. However, it should be noted that the highest dose of pentobarbitone used *in vitro* (5×10^{-4}M) inhibited the response to field stimulation of canine trachealis muscle by 64% whereas, *in vivo*, in dogs anaesthetized with this anaesthetic, histamine aerosol failed to produce a reflex bronchoconstriction. Further the increase in R_L produced by electrical stimulation of the cervical vagosympathetic nerves in these animals was only one tenth of that produced in dogs anaesthetized with chloralose.

We offer the explanation, therefore, that pentobarbitone inhibits reflex bronchoconstriction principally, but not solely, by an action on the parasympathetic postganglionic nerve fibres, or endings in the lung. We cannot say whether this conclusion extends to other species.

We would like to thank Professor J.G. Widdicombe for his help and advice in the preparation of this paper. We are grateful to Mr K. Vendy and Mrs L.M. Axelrad for their excellent technical assistance.

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(Received February 3, 1977.
Revised March 23, 1977)

Br. J. Pharmac. (1977), **61**, 257–262

THE EFFECTS OF SODIUM CROMOGLYCATE ON HISTAMINE AEROSOL-INDUCED REFLEX BRONCHOCONSTRICTION IN THE ANAESTHETIZED DOG

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1 The effects have been studied of sodium cromoglycate (SCG), given by aerosol or intravenously, on reflex bronchoconstriction induced by histamine aerosol in the anaesthetized dog.

2 Four breaths of an aerosol generated from a 2% solution of SCG significantly inhibited the vagally mediated increase in total lung resistance (R_L) produced by histamine.

3 SCG given intravenously as bolus injections (5–500 μg/kg) produced a dose-dependent reversal of a sustained reflex bronchoconstriction induced by histamine aerosol. Propranolol (500 μg/kg) did not prevent this reversal.

4 SCG did not inhibit the increase in R_L produced by supramaximal electrical stimulation of a vagus nerve.

5 The possibility is discussed that SCG may reduce the activity of lung irritant receptors in the anaesthetized dog.

Introduction

Sodium cromoglycate (SCG) is a drug of proven worth in the treatment of certain types of asthma (Editorial, British Medical Journal, 1972). Evidence obtained from *in vitro* and *in vivo* experiments has led to the proposal that SCG acts by the temporary stabilization of the mast cell so preventing the release of mediators (Cox, Beach, Blair, Clarke, King, Lee, Loveday, Moss, Orr, Ritchie & Sheard, 1970). We have investigated another possible explanation for the action of SCG in the lung. Using a canine model of reflex bronchoconstriction we have assessed the ability of this drug, given by aerosol or intravenously, to inhibit or reverse bronchoconstriction mediated via the vagus nerves. Some of the results of this study have been communicated to the British Pharmacological Society (Jackson & Richards 1976).

Methods

Beagle dogs (9–12 kg) of either sex were initially sedated with thiopentone sodium (5–10 mg/kg i.v.) and then anaesthetized with chloralose (80 mg/kg i.v.). The animals were maintained at a light level of surgical anaesthesia by giving supplementary doses of chloralose 10–15 mg/kg every 15 min via a cannula inserted in the right saphenous vein.

The trachea was cannulated just below the cricoid cartilage with a plastic cannula (1 cm internal diameter and 3 cm long with a resistance of 0.04 kPa l^{-1} s) and the animals were ventilated with air at constant pressure (0.98 kPa) using a Bird Mark VII ventilator. The lungs were inflated to 1.96 kPa transpulmonary pressure (TPP) after severe histamine-induced falls in lung compliance and also periodically to reverse the gradual decrease in lung volume, which occurs during positive pressure ventilation. A thoracotomy was routinely performed.

A catheter was inserted in the muscularis branch of the right femoral artery for recording blood pressure with a Statham P23 Db pressure transducer. Heart rate was derived from the blood pressure signal using a Devices ratemeter. Catheters were placed in the left saphenous vein and the muscularis branch of the left femoral artery for the injection of drugs and the removal of arterial blood for blood-gas analysis respectively.

Both cervical vagosympathetic nerves were carefully exposed and placed on silver thermodes, 2.5 cm long, through which water at 0.5°C could be circulated. When the nerves were cooled, chilled saline (0.9% w/v NaCl solution) swabs were placed on the nerves which passed over the thermodes.

In three dogs both cervical vagosympathetic nerves were cut and the sheath removed from the peripheral cut end of the right vagosympathetic nerve with the aid of a dissecting microscope. The sympathetic fibres were split away and the remaining parasympathetic nerve placed on bipolar electrodes under warm liquid paraffin. The electrodes were connected to a square wave stimulator (Devices) which was set to deliver pulses of 1 ms at a frequency of 20 Hz. The voltage was adjusted to obtain a maximal response on R_L.

The body temperature of the dogs was maintained at 38.5°C with a thermostatically controlled heating blanket in conjunction with a rectal thermocouple.

The partial pressures of oxygen (PO_2) and carbon dioxide (PCO_2) in the blood and the blood pH were monitored regularly with a Radiometer ABL 1 acid-base analyser. By adjusting the ventilation of the animal at the beginning of the experiment, or during the control periods, it was possible to maintain $PO_2 > 13.3$ kPa, PCO_2 between 3.32 kPa and 5.32 kPa and the pH between 7.36 and 7.46 Pa.

Air flow rate was measured with a Fleisch pneumotachograph (Type 0; 9.8 Pa = 43.16 ml/s) and a Furness Controls micromanometer (100–0–100 Pa) and tidal volume obtained by electrical integration of the flow signal with a Devices integrator. TPP was measured by a Furness Controls micromanometer (1–0–1 kPa) one side of which was connected to the tracheal cannula and the other side of which was left open to the atmosphere. All recordings were displayed on a Devices M19 recorder. Total lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were measured by a manual graphic method using the displayed signs of flow, volume and TPP (Amdur & Mead, 1958). The respiratory computer described by Carney, Pugh & Sheard (1972) was also used for the calculations of R_L and C_{dyn}, its displayed output being calibrated and checked for accuracy by comparison with simultaneous manual determinations of R_L and C_{dyn}. The computer was accurate to ± 0.02 kPa l^{-1} s for R_L and ± 6.2 ml kPa^{-1} for C_{dyn}.

Aerosols were generated on inspiration only using a Vaponefrin inhalajet nebuliser modified to deliver an aerosol containing mainly large particles (12.8 µm).

Experimental procedure

Aerosol study. Bronchoconstriction was produced by allowing the dogs to inhale 4 breaths of histamine aerosol. The concentration of histamine solution was selected from a range of 0.0625 to 0.25% such that a change in R_L of 0.98–1.96 kPa l^{-1} s was produced. At the peak of the change in R_L both cervical vagi were cooled to 0.5°C to determine the reflex component of the bronchoconstriction.

Histamine challenges were given every 30 min until 4 consistent bronchoconstrictions had been produced. Four breaths of an aerosol of SCG generated from either 1% or 2% solutions were then given 10 min before the next histamine challenge. The SCG aerosol was generated by a nebuliser similar to that used to generate the histamine aerosols. Further control histamine challenges were given every 30 min for at least 2 hours.

Solutions of SCG greater than 2% concentration are viscous and were not used in this study since difficulty was encountered in the generation of aerosols.

Intravenous study. Two to four breaths of histamine aerosol of appropriate concentration were given to produce an increase in R_L greater than 0.98 kPa l^{-1} s.

The reflex nature of this resistance change was checked by rapidly cooling the cervical vagi. The nerves were then quickly rewarmed to 38.5°C to re-establish the bronchoconstriction and when the change in R_L was seen to be stable, SCG was given intravenously as a bolus injection. At least 30 min were allowed between histamine challenges.

The effects of sodium cromoglycate given by aerosol and intravenously on the increase in R_L produced by stimulation of the right vagus nerve. The right cervical vagus nerve was stimulated every 15 min for 20 s until 3 consistent increases in R_L were seen. Ten minutes before the next stimulation 4 breaths of an aerosol of SCG generated from a 2% solution were given. Intermittent nerve stimulation was continued for at least a further 30 minutes.

In the same dogs, at least 2 h later, the right cervical vagus nerve was again stimulated until three consistent increases in R_L were seen; SCG 100 µg was then given intravenously 70 s before the next stimulation.

Drugs

The drugs used were: thiopentone sodium (Intraval, May and Baker Limited); α-chloralose (Koch-Light Laboratories); sodium cromoglycate (Fisons Limited); histamine acid phosphate (BDH); isoprenaline hydrochloride (Suscardia, Pharmax); propranolol hydrochloride (Inderal, ICI).

Drug solutions were freshly prepared in isotonic saline and concentrations are given in terms of the bases.

Results

Aerosol study

The results of this study are shown in Figure 1. An aerosol of SCG generated from a 1% solution produced some reduction in the vagally mediated increase in R_L induced by histamine aerosol, although this was not statistically significant ($P > 0.05$). However, an aerosol of SCG generated from a 2% solution produced a significant reduction ($P < 0.05$) in

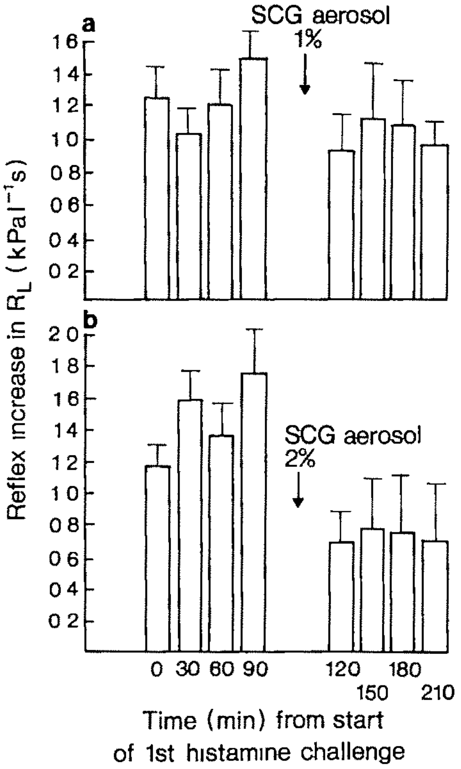

Figure 1 The effect of 4 breaths of an aerosol of sodium cromoglycate (SCG) generated in (a), from a 1% w/v solution and in (b), from a 2% w/v solution on the reflex increases in total lung resistance (R_L) induced by histamine aerosol. Bars are s.e. mean; $n=6$ for 1% aerosol and 4 for 2% aerosol.

the vagally mediated change in R_L and this reduction lasted for at least 2 hours. In one dog the response to histamine aerosol reverted to control values after 3 hours. Statistical comparison was made with the response prior to SCG.

SCG given by aerosol did not produce any cardiovascular changes.

In 5 dogs, 4 breaths of a saline aerosol were given as a control. This did not affect the resting values of R_L or C_{dyn}, nor did it affect the response of the airways to subsequent histamine challenges.

Intravenous study

The effect of 500 µg/kg SCG intravenously on a sustained bronchoconstriction is shown in Figure 2. The effects of 100, 50, 25, 10 and 5 µg/kg SCG (i.v.) on similar reflex bronchoconstrictions have also been investigated and the results are shown in Table 1, together with the changes in mean arterial pressure. SCG 5–50 µg/kg produced dose-related reversals of the reflex bronchoconstriction; 100% reversal occurring with 50, 100, and 500 µg/kg. The maximal effect of SCG occurred 70 s after injection and lasted for at least 5 minutes. In these experiments the duration of action of SCG could not be accurately determined because the falls in C_{dyn} induced by histamine necessitated reversal by hyperinflation.

SCG 5 µg/kg did not affect mean arterial blood pressure, but higher doses caused significant falls.

To eliminate the possibility that SCG was reversing the reflex bronchoconstriction by releasing endogenous sympathomimetic amines, we investigated the action of SCG in the presence of the β-adrenoceptor blocking drug, propranolol. In 3 dogs a reflex bronchoconstriction was produced and a partial reversal obtained by giving SCG 25 µg/kg intravenously. The remaining increase in R_L was then reversed with isoprenaline (500 ng/kg). Propranolol (500 µg/kg i.v.) was then given and 30 min later a reflex bronchoconstriction established. When SCG 25 µg/kg was given (i.v.) a partial reversal of the bronchoconstriction was again seen. However, the remaining increase in R_L was not reversed by isoprenaline. The records of one such investigation are shown in Figure 3.

The effects of sodium cromoglycate on the increases in R_L produced by stimulation of the right vagus nerve

The results of this study are shown in Table 2. Neither four breaths of an aerosol of SCG generated from a 2% solution nor 100 µg/kg SCG given intravenously affected the increases in R_L produced by electrical stimulation of the right vagus nerve; the resting values of R_L were similarly unaffected.

Electrical stimulation of the right cervical vagus caused cardiac arrest with subsequent 'vagal escape'.

Discussion

Four breaths of an aerosol of SCG generated from a 2% solution significantly reduced the reflex bronchoconstriction to histamine aerosol, and SCG, 5–500 µg/kg intravenously produced dose-dependent reversals of sustained reflex bronchoconstrictions. SCG did not affect the bronchoconstriction produced by the direct electrical stimulation of the right vagus nerve, nor did it affect the basal total lung resistance. Although it is reasonable to propose that the modes of action of the drug, given by aerosol and by the intravenous route were the same, the results of this study cannot establish this. One significant difference between the intravenous route and aerosol routes of administration is that in the anaesthetized dog SCG given intravenously, as a bolus injection in doses of 10 µg/kg or more, produces a fall in arterial

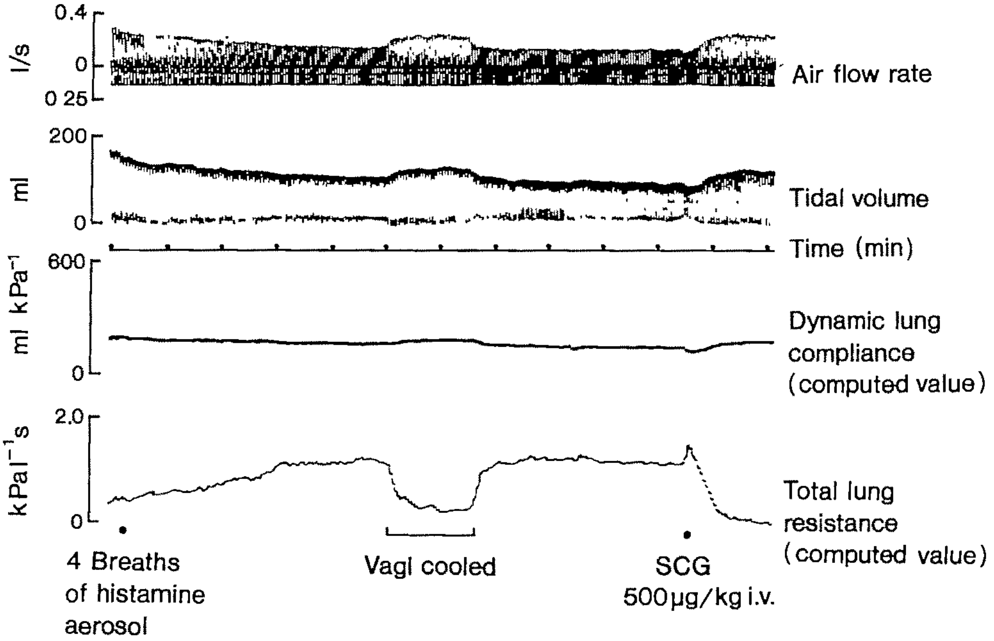

Figure 2 The effects of sodium cromoglycate (SCG) 500 µg/kg intravenously on a sustained reflex bronchoconstriction induced in a dog anaesthetized with chloralose.

blood pressure and heart rate (Cox *et al.*, 1970). This response depends on the vagus nerves and is similar to the response to the veratrum alkaloids described by Bezold & Hirt (1867) and Jarisch & Richter (1939). It was thought possible that the fall in blood pressure produced by SCG in the present experiments, when it was given intravenously, may have caused the release of endogenous sympathomimetic amines and that these could have been responsible for the reversal of the bronchoconstriction. However, SCG was still active in dogs which had received 500 µg/kg propranolol and in which the airways did not respond to isoprenaline (Figure 3).

These observations coupled with the result that SCG was active when given by aerosol, when a reflex fall in blood pressure and heart rate did not occur, make it unlikely that the cardiovascular changes produced by SCG are in any way connected with the reversal of the reflex bronchoconstriction. It therefore seems most likely that the mechanisms of the inhibition or reversal of the bronchoconstriction by SCG were the same whichever route of administration was employed.

Cox *et al.* (1970) reported that SCG does not act directly on airways smooth muscle and is not a pharmacological antagonist of the actions of histamine or acetylcholine. Although an action on the CNS cannot be entirely ruled out, SCG has been administered by a variety of routes (i.v., i.m., inhalation, conjunctival sac, intranasal) to the dog in single and multiple doses ranging from 1–20 mg/kg and the highest amount of SCG ever found in the total brain tissue in all these studies was only 0.09% of the dose. Even a proportion of this was probably due to a certain amount of contamination of the brain tissue by blood (personal communication from Dr B. Clark, the Department of Metabolic Studies, Fisons Limited). Further indirect evidence for lack of CNS penetration by SCG is given by Cox *et al.* (1970) and Ashton, Clark, Jones, Moss, Neale & Ritchie (1973). Our experiments further showed that SCG given by aerosol or intravenously did not modify the effects of direct electrical stimulation of the efferent vagus nerves to the lung. It is likely, therefore, that SCG reduced the reflex bronchoconstriction to histamine by an action on the afferent arm of the reflex. Mills, Sellick & Widdicombe (1969), Gold, Kessler & Yu (1972) and Sampson & Vidruk (1975) have suggested that the lung irritant receptors could participate in the afferent limb of histamine-induced reflex bronchoconstriction. It is possible that SCG might reduce the activity of lung irritant receptors; such an action would explain the inhibition or reversal of a reflex bronchoconstriction seen in these experiments. Only by measuring the effect of SCG on action potentials from single vagal fibres originating from lung irritant receptors could this hypothesis be confirmed.

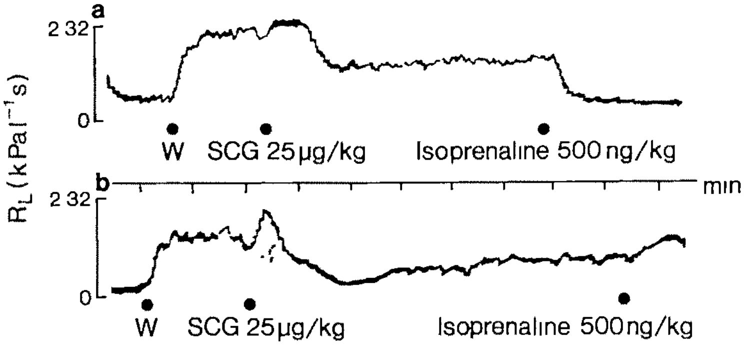

Figure 3 The effects of sodium cromoglycate (SCG) 25 µg/kg intravenously and isoprenaline 500 ng/kg intravenously on a sustained reflex bronchoconstriction; (a) in the absence of propranolol and (b) in the presence of 500 µg/kg propranolol. The records were taken from the same dog. Histamine was administered by aerosol prior to this record. The vagi were cooled to confirm the reflex component of the bronchoconstriction and were rewarmed at W after which bronchoconstriction became re-established.

Table 1 The effects of intravenous sodium cromoglycate (SCG) on histamine-induced reflex bronchoconstriction and on mean arterial blood pressure

Dose of SCG (µg/kg i.v.)	*% reversal of histamine induced bronchoconstriction mean ± s.e. mean*	*Fall in mean arterial blood pressure (mmHg) mean ± s.e. mean*	n
5	17 ± 2.1	No change	4
10	26 ± 4.0	36.6 ± 6.4	4
25	51.3 ± 9.7	48.8 ± 13.4	4
50	100	28.3 ± 14.8	3
100	100	70.0 ± 5.8	3
500	100	66.0 ± 13.0	3

Table 2 The effects of sodium cromoglycate (SCG) given by aerosol or intravenously on the increase in R_L produced by electrical stimulation of the right vagus

Time (min) of event	*Resting R_L (kPa l^{-1} s)*	*Increase in R_L (kPa l^{-1} s) produced by *stimulation of R. cervical vagus*
0	0.17 ± 0.03	1.24 ± 0.28
15	0.17 ± 0.02	1.23 ± 0.29
20	*4 breaths of an aerosol of SCG generated from a 2% solution*	
30	0.18 ± 0.03	1.28 ± 0.29
45	0.17 ± 0.03	1.13 ± 0.33
	Intravenous SCG	
0	0.15 ± 0.02	0.63 ± 0.07
13 min 50 s	100 µg/kg SCG i.v.	
15	0.15 ± 0.02	0.63 ± 0.08

Values are mean ± s.e. mean. $n=3$ (aerosol); $n=5$ (i.v.). * Stimulus parameters were 20 Hz, 1 ms, supramaximal voltage for 20 seconds.

There has been some controversy over the role of the vagus nerves in allergic bronchoconstriction (Gold, 1973). Widdicombe and his colleagues have studied the role of vagally mediated reflexes in the response to systemically administered antigen in guinea-pigs and rabbits. Using single fibre recordings from lung irritant receptors and the analysis of lung dynamics during anaphylaxis, they demonstrated conclusively that the vagus plays an important role in the production of anaphylactic bronchoconstriction (Karczewski & Widdicombe, 1969; Mills, Sellick & Widdicombe, 1969; Mills & Widdicombe, 1970). It is likely, therefore, that a drug which reduced the sensitivity of lung irritant receptors in man would go some way to reliving an asthmatic attack. The results of our experiments suggest that SCG may reduce the activity of lung irritant receptors in the anaesthetized dog. This action may be relevant to the way in which the drug acts in asthma.

We would like to thank Professor J.G. Widdicombe for his help and advice in the preparation of this paper. We gratefully acknowledge the excellent technical assistance of Mr K. Vendy.

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(Received February 3, 1977.
Revised March 23, 1977)

Br. J. Pharmac. (1977), **61**, 263–269

RELAXATION OF ISOLATED AORTA OF THE RABBIT BY PICOLINIC ACIDS

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1 When the isolated thoracic aorta of the rabbit was contracted with prostaglandin $F_{2\alpha}$, 5-alkylpicolinic acids produced dose-dependent relaxations.

2 Picolinic acid, 2,5-pyridinedicarboxylic acid and 5-acetylpicolinic acid which do not have the 5-alkyl residue failed to relax blood vessels.

3 The vascular relaxation was dependent on the number of carbon atoms in the 5-alkyl compounds.

4 Relaxations which occurred with 5-alkylpicolinic acids were not affected by pretreatment with either propranolol or atropine.

5 It is concluded that the 5-alkyl residue is necessary for the vascular relaxation with 5-alkylpicolinic acid and that it was not produced by stimulation of β-adrenoceptors or cholinoceptors but rather through an activation of the basic process.

Introduction

Fusaric (5-butylpicolinic) acid (FA) is a potent inhibitor of a purified bovine adrenal dopamine β-hydroxylase [EC: 1,14,2,1] (DBH) (Hidaka, Nagatsu, Takeya, Takeuchi, Suda, Kojiri, Matsuzaki & Umezawa, 1969) and an effective antihypertensive and hypotensive agent in man (Terasawa & Kameyama, 1971) and other mammals (Hidaka & Takeya, 1972; Hidaka, Nagasaka & Takeda, 1973; Hidaka, Hara, Harada, Hashizume & Yano, 1974a). A significant reduction in blood pressure was demonstrated in both spontaneously hypertensive rats (SHR) and normotensive rabbits after intravenous and intraperitoneal injections or oral administration of fusaric acid or 5-(3′,4′-dibromobutyl)picolinic acid (dibromorfusarate Br_2FA) (Hidaka & Takeya, 1972; Hidaka, Hara, Harada & Asano, 1974b).

Thus, it is conceivable that the reduction in blood pressure with FA is the result of reduced formation of tissue noradrenaline content through inhibition of DBH (Hidaka, Shoka, Hashizume, Takemoto & Yamamoto, 1975). On the other hand, decrease in blood pressure with FA is observed immediately (1–2 min) after intraveneous injection (Hidaka *et al.*, 1974b). Such a rapid hypotensive effect of FA cannot be explained only by decrease in tissue noradrenaline through inhibition of DBH. We also found that FA does not release tissue catecholamines (Hidaka *et al.*, 1974a). Therefore, the possibility that FA lowers the blood pressure through mechanisms other than inhibition of DBH or release of catecholamines must be considered. Recently, we demonstrated that FA causes a relaxation of rabbit arterial strips contracted with either prostaglandin $F_{2\alpha}$ or KCl and also inhibits the contraction induced by various agonists (Asano & Hidaka, 1975; Hidaka & Asano, 1976).

The vascular relaxation produced by various derivatives of picolinic acid and pyridine has been studied. The structure-activity relationship between the derivatives of FA was demonstrated by using several analogues of FA such as pyridine-monocarboxylic acid, pyridine-dicarboxylic acids, 5-acetylpicolinic acid and 5-alkylpicolinic acids.

Methods

Albino rabbits of either sex weighing 1.9–2.5 kg were killed by a blow on the head and exsanguinated. The thoracic aorta (2.5–5.0 mm outside diameter) was quickly excised and after removal of adventitial connective tissue, the aorta was cut to make a spiral strip, 2–3 mm in width and 25–35 mm in length (Lewis & Koessler, 1927; Furchgott & Bhadrakom, 1953). The spiral strip was fixed vertically between hooks in a water-jacketed tissue bath containing 40 ml of modified Ringer Locke solution having the following composition (mM): NaCl 140.9, KCl 5.6, $CaCl_2 2H_2O$ 2.2, $NaHCO_3$ 14.9 and dextrose 5.6. Solutions were maintained at 37 ± 0.5°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The upper end of the strip was connected to the lever of a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co., Tokyo, Japan) by a silk thread. An initial resting tension of 1 g was applied to the aorta. Before the experiments were started, preparations were allowed to equilibrate for 1 h in the bathing solution. During this period, the solutions were replaced every 20 minutes.

Cumulative dose-response curves for vascular responses to the agonists were obtained by increasing the concentrations by a factor of about 3, while the previous dose remained in contact with the tissue (van Rossum, 1963). Each concentration was added only after the effects of the previous concentration had reached a maximum and remained constant. Maximum responses obtainable were assumed when a concentration of 3×10^{-3} M of each agonist was added. To compare agonists which relax blood vessels, papaverine in a concentration of 1×10^{-4} M was added at the end of each series of experiments and relaxation induced by papaverine was taken as 100% (Toda, 1974; Buckner & Saini, 1975). Stock solutions of drugs were added directly to the bathing solution in a volume of 0.4 ml to give the final concentrations desired. Only one cumulative dose-response curve to a compound was obtained from a single preparation. Usually paired strips from the same animal were given different treatments and, in drug antagonism studies, one strip was used to obtain the control dose-response curve. Effects of various blocking agents such as propranolol, atropine, aminophylline, haloperidol and ouabain on relaxations induced by picolinic acids were determined with these blocking agents before the addition of prostaglandin $F_{2\alpha}$. Whenever an ED_{50} value was determined, responses to the agonists were calculated as a percentage of the maximum relaxation obtained with that agonist. The ED_{50} value was obtained visually from a plot of percentage relaxation *v* log concentration of the agonist and expressed as the negative logarithm (−log molar ED_{50}). Results shown in the text, tables and figures are expressed as the mean value ± s.e. Comparison of the results was made by Student's *t*-test. Statistical significance was assumed when $P < 0.05$.

Drugs and chemicals

The following drugs were used: (±)-propranolol hydrochloride (Sigma), atropine sulphate (Katayama), aminophylline, haloperidol (Serenace, Speeling Dainippon Pharmaceutical Co., Ltd.), ouabain (Merck), prostaglandin $F_{2\alpha}$ (Prostarmon F, Ono Pharmaceutical Co., Ltd.), papaverine hydrochloride (Katayama), (−)-isoprenaline hydrochloride (Sigma), $N^6,O^{2'}$dibutyryl cyclic adenosine 3′:5′-monophosphoric acid monosodium salt (Boehringer), picolinic acid sodium salt (Tokyo Kasei Kogyo Co., Ltd.), nicotinic acid sodium salt (Tokyo Kasei Kogyo Co., Ltd.), isonicotinic acid (Wako), 2-aminopyridine (Wako), 2,3-pyridinedicarboxylic acid (Wako) and 2,5-pyridinedicarboxylic acid (Wako). 2,4-Pyridinedicarboxylic acid, 2,6-pyridinedicarboxylic acid, 5-acetylpicolinic acid, 5-methylpicolinic acid, 5-butylpicolinic acid (FA), 5-pentylpicolinic acid, 5-(monobromomethyl)picolinic acid, 5-(3′,4′-dibromobutyl)picolinic acid (Br_2FA), 5-(*N,N*-dimethylthiocarbomoylthiomethyl)picolinic acid (YP-279) and 5-(*N,N*-dimethylthiocarbamoylthiomethyl)picolinic acid monomethylamide (YP-614) were synthesized by Dr I. Matsumoto of Banyu Pharmaceutical Co., Ltd. Picolinic acids tested are shown in Table 1.

Picolinic acid derivatives were prepared in distilled water and pH was adjusted to 7.4 with sodium hydroxide. Other drugs (with the exception of papaverine, which was prepared in 0.9% w/v NaCl solution (saline)) were prepared daily in Ringer Locke solution and kept on ice during the course of the experiment.

Results

Relaxation induced by picolinic acid derivatives in aortic strips

The addition of prostaglandin $F_{2\alpha}$ in a concentration of 5×10^{-7}M caused a sustained contraction in aortic strips (Figure 1a). When preparations were contracted with 5×10^{-7} M prostaglandin $F_{2\alpha}$, different degrees of tension (0.1–2.4 g) were obtained. The mean value of the tension developed in thoracic aortae was 991 ± 97 mg ($n = 99$). Preparations thus contracted showed good relaxation in response to vasodilators. The addition of 5-pentylpicolinic acid in concentrations ranging from 3×10^{-6} M to 3×10^{-3} M elicited a dose-dependent relaxation (Figure 1b). The slope of the dose-response curve for relaxation to 5-pentylpicolinic acid remained the same provided that the initial tension developed by the prostaglandin $F_{2\alpha}$ lay between 0.5 and 2.4 grams. The slope of the curve was reduced when the initial tension was less than 0.4 gram. In another experiment, the relationship between the concentration of prostaglandin $F_{2\alpha}$ and the 5-pentylpicolinic acid-induced relaxation was examined. When the preparation was contracted with doses of prostaglandin $F_{2\alpha}$(5×10^{-7} M, 1×10^{-6} M and 2×10^{-6} M) the maximum relaxation obtained with 5-pentylpicolinic acid was the same; but concentrations of 5×10^{-8} M and 5×10^{-6} M produced different maximal effects. Consequently, prostaglandin $F_{2\alpha}$ (5×10^{-7}M) was usually used to produce a tension of approximately 1.0 gram. If this concentration produced a tension of less than 1.0 g, it was raised to 1×10^{-6} M and in a few cases to 2×10^{-6} M until a tension within the range 1.0–2.0 g was produced.

The additions of 5-methylpicolinic acid, 5-butylpicolinic acid (FA), 5-pentylpicolinic acid, 5-(bromomethyl)picolinic acid, 5-(3′,4′-dibromobutyl-picolinic acid (Br_2FA), 5-(*N,N*-dimethylthiocarbamoylthio-

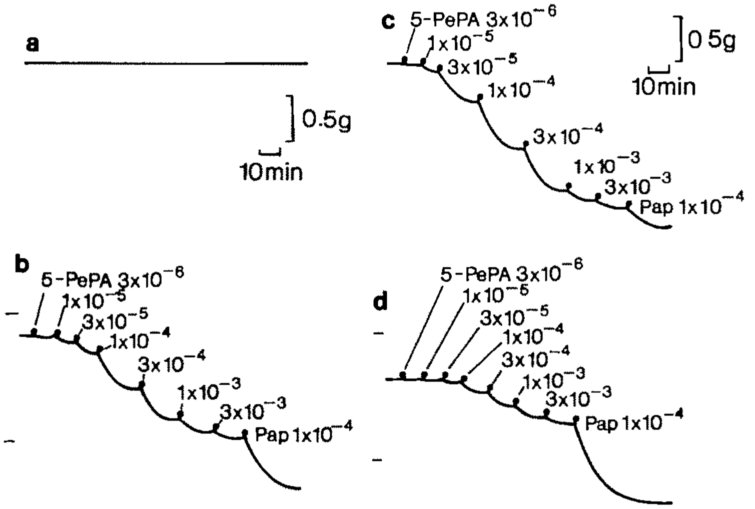


Figure 1 Responses of a strip of rabbit thoracic aorta to 5-pentylpicolinic acid. The preparations were contracted with prostaglandin $F_{2\alpha}$ 5×10^{-7} M. Horizontal line on left of each tracing indicates the level of tension before the addition of prostaglandin $F_{2\alpha}$. (a) Control without 5-pentylpicolinic acid showing that the contraction of the preparation by prostaglandin is sustained for at least 2 hours. (b) The 5-pentylpicolinic acid (5-PePA) induced dose-dependent relaxation of the preparation contracted with prostaglandin $F_{2\alpha}$. Further relaxation induced by papaverine 1×10^{-4} M (Pap). (c) Relaxation induced by 5-pentylpicolinic acid was enhanced by pretreatment with haloperidol and in (d) was attenuated by ouabain. Haloperidol and ouabain were preincubated with the strips for 10 min before the addition of prostaglandin $F_{2\alpha}$. Molar concentrations are shown.

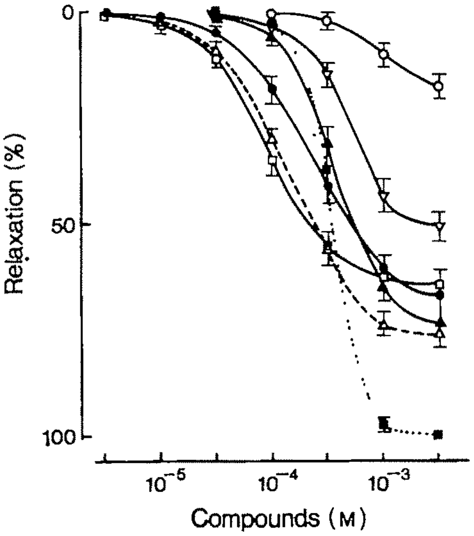


Figure 2 Dose-relaxation curves for 5-alkylpicolinic acids in strips of thoracic aorta. Tension induced by prostaglandin $F_{2\alpha}$ (5×10^{-7} M) was 1025×77 mg ($n=19$) and papaverine (1×10^{-4} M)-induced maximum relaxation was 1461 ± 114 mg ($n=19$). The relaxation induced by 1×10^{-4} M of papaverine was taken as 100%. Vertical bars represent s.e. Figures in parentheses indicate the number of preparations used. (○) 5-Methylpicolinic acid; (●) 5-butylpicolinic acid; (□) 5-pentylpicolinic acid; (■) 5-(bromomethyl)picolinic acid; (△) 5-(3′,4′-dibromobutyl)picolinic acid; (▲) 5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid; ( ) 5-(*N*,*N*-dimethylthiocamoylthiomethyl)picolinic acid monoethylamide.

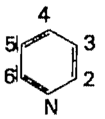
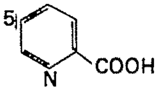
methyl)picolinic acid (YP-279) and 5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid monomethylamide (YP-614) caused dose-dependent relaxations (Figure 2). However, pyridinemonocarboxylic acids (such as picolinic acid, nicotinic acid and isonicotinic acid), pyridinedicarboxylic acids (such as 2,3-, 2,4-, 2,5-, and 2,6-pyridinedicarboxylic acid) and other compounds (such as 2-aminopyridine, 5-acetylpicolinic acid) failed to relax the aorta. The average percentage relaxation produced by picolinic acid derivatives (1 mM) and the negative logarithm of the molar concentrations for the ED_{50} (−log molar ED_{50})

are shown in Table 1. Thus the 5-alkyl residue was necessary for the vascular relaxation produced by 5-alkylpicolinic acids and the 2-carboxyl groups also contributed. According to the ED_{50} values obtained, the potency of the vascular relaxing response of 5-alkylpicolinic acids was in the order of 5-pentylpicolinic acid > Br_2FA > FA > YP-279 > 5-(bromomethyl) picolinic acid > YP-614 > 5-methylpicolinic acid.

Effects of propranolol, atropine, aminophylline, haloperidol and ouabain on 5-alkylpicolinic acids-induced relaxations

The relaxation of the aortic strip produced by 5-alkylpicolinic acids was not affected by pretreatment with propranolol 5×10^{-6} M, atropine 1×10^{-5} M or by aminophylline 3×10^{-4} M (an adenosine antagonist) (Table 2). However dose-relaxation curves for FA, 5-pentylpicolinic acid, Br_2FA, YP-279, YP-614 and 5-methylpicolinic acid shifted to the left after pretreatment with 1×10^{-5} M haloperidol (Figure 1c and Table 3). When the preparations were pretreated with ouabain 2×10^{-7} M or 1×10^{-6} M, dose-relaxation curves for 5-alkylpicolinic acids were inhibited dose-dependently (Figure 1d and Table 3). Thus the relaxation caused by 5-alkylpicolinic acids was enhanced by haloperidol and inhibited by ouabain, while the ED_{50} values were not affected (Table 3). Relaxation of the thoracic aorta by isoprenaline, $N^6,O^{2\prime}$dibutyryl cyclic adenosine 3′:5′-monophosphoric acid (dibutyryl cyclic AMP) or by aminophylline, was also enhanced by haloperidol and attenuated by ouabain (Table 3). Papaverine

Table 1 Arterial strip relaxation induced by picolinic acid and its derivatives

Compounds	*Pyridine* 	n	% *Relaxation**	*—Log molar ED_{50}*†
Picolinic acid	2: COOH	10	3.1 ± 1.1	
Nicotinic acid	3· COOH	9	3.6 ± 1.4	
Isonicotinic acid	4: COOH	8	1.4 ± 0.6	
2-Aminopyridine	2: NH_2	10	0.3 ± 0.2	
2,3-Pyridinedicarboxylic acid	2,3: COOH	8	5.0 ± 2.1	
2,4-Pyridinedicarboxylic acid	2,4: COOH	8	2.0 ± 1.0	
2,5-Pyridinedicarboxylic acid	2,5: COOH	9	3.3 ± 0.9	
2,6-Pyridinedicarboxylic acid	2,6: COOH	8	4.0 ± 2.1	
Picolinic acid				
5-Acetylpicolinic acid	5· $COCH_3$	8	2.7 ± 1.0	
5-Methylpicolinic acid	5· CH_3	8	10.2 ± 2.4	
5-Butylpicolinic acid	5: $CH_2CH_2CH_2CH_3$	19	60.1 ± 2.6	3.67 + 0.04
5-Pentylpicolinic acid	5: $CH_2CH_2CH_2CH_2CH_3$	9	62.1 ± 3.4	4.07 + 0.04
5-(Monobromomethyl)picolinic acid	5: CH_2Br	19	97.0 ± 1.0	3.40 + 0.06
5-(3′,4′-Dibromobutyl)picolinic acid	5: $CH_2CH_2CHBrCH_2Br$	13	73.2 ± 1.4	3.87 + 0.05
5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid	5: $CH_2SCSN(CH_3)_2$	11	65.4 ± 2.6	3.43 + 0.03
5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid monomethylamide	5: $CH_2SCSN(CH_3)_2$ 2: $CONHCH_3$	15	43.1 ± 3.8	3.33 + 0.01

* Relaxation produced by a concentration of 1 mM expressed as a percentage of the maximum response produced by papaverine (1×10^{-4} M) which was taken as 100%. Tension induced by prostaglandin $F_{2\alpha}$ (5×10^{-7}M) was 1039 ± 61 mg ($n = 67$).

† The ED_{50} value was obtained visually from a plot of percentage relaxation *v* log concentration of the compound and expressed as the negative logarithm (—log molar ED_{50}).

n indicates the number of preparations used. Data are expressed as mean ± s.e. mean.

(1×10^{-4} M)-induced maximum relaxation was not affected by pretreatment with haloperidol, ouabain, propranolol, atropine or aminophylline.

Discussion

All 5-alkylpicolinic acids tested produced relaxation of the rabbit aorta. 5-Methylpicolinic acid, FA- and 5-pentylpicolinic acid caused a dose-dependent relaxation of the thoracic aorta and the potency depended on the number of carbon atoms in the 5-alkyl residue. 5-Pentylpicolinic acid and FA produced a greater relaxation than did 5-methylpicoloinic acid. The addition of a large molecule to the 5-alkyl residue of 5-alkylpicolinic acid potentiated the response. For example 5-(monobromomethyl)picolinic acid and 5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid (YP-279) induced a more potent relaxation of the aorta than did 5-methylpicolinic acid. 5-(3′,4′-Dibromobutyl)picolinic acid (Br_2FA) proved to be more potent than FA in inducing vascular relaxation. However, picolinic acid, 2,5-pyridinedicarboxylic acid and 5-acetylpicolinic acid which do not have the 5-alkyl residue failed to relax the blood vessels. From these observations, it is presumed that the 5-alkyl residue is necessary for vascular relaxation with 5-alkylpicolinic acid.

Suda, Takeuchi, Nagatsu, Matsuzaki, Matsumoto & Umezawa (1969) discussed the relationship between the inhibition of DBH by 5-alkylpicolinic acids and their hypotensive effects. These workers demonstrated that picolinic acid and all 5-alkylpicolinic acids inhibit bovine adrenal DBH and that all of them had hypotensive effects. The degree of DBH inhibition is dependent on the number of carbon atoms in the 5-alkyl group and 5-butyl and 5-pentyl compounds are more potent than the others. When the carbon atoms in the 5-alkyl residue are increased to 4 or 5, the hypotensive effect becomes stronger, thus 5-butyl- and 5-pentyl-picolinic acids were more potent than 5-propionyl-, 5-ethyl- and 5-methyl-picolinic acids. It is interesting that the hypotensive effects of picolinic acid derivatives appear to correlate with both their vasodilator actions and their DBH inhibitory activities.

In our previous studies (Asano & Hidaka, 1975; Hidaka & Asano, 1976), 5-butylpicolinic acid (FA), one of the 5-alkylpicolinic acids was observed to have a direct relaxant action on blood vessels. FA decreased the contractor responses elicited with noradrenaline, histamine, 5-hydroxytryptamine, acetylcholine, angiotensin II and KCl in helical strips of rabbit aorta (Ao) and superior mesenteric (Sm), carotid (Cd), renal (R) and femoral (Fe) arteries. When blood vessels were contracted with either prostaglandin $F_{2\alpha}$ or KCl, FA caused a relaxation of Ao, Sm, Cd, R and Fe arteries in a dose-dependent fashion. FA has an anti-hyper-

Table 2 Effects of propranolol, atropine and aminophylline on the vascular relaxation produced by picolinic acid derivatives

		n	*Control*	n	*Propranolol‡ (5×10^{-6} M)*	n	*Atropine‡ (1×10^{-5} M)*	n	*Aminophylline‡ (3×10^{-4} M)*
% Relaxation*	FA	19	60.1 ± 2.6	8	61.3 ± 5.3	8	54.6 ± 4.9	8	55.4 ± 4.7
	5-pentyl PA	9	62.1 ± 3.4	8	65.7 ± 4.2	8	57.6 ± 4.7	8	51.3 ± 4.8
	BR_2FA	13	73.2 ± 1.4	8	78.6 ± 2.8	8	75.1 ± 3.5	8	69.6 ± 2.5
	YP-279	11	64.5 ± 2.6	8	63.0 ± 4.6	8	58.3 ± 5.6	8	56.0 ± 4.8
	YP-614	15	43.1 ± 3.8	8	53.2 ± 6.0	8	48.5 ± 2.9	8	42.5 ± 4.8
−Log molar ED_{50}†	FA	19	3.76 ± 0.04	8	3.67 ± 0 05	8	3.81 ± 0.06	8	3.85 ± 0.06
	5-pentyl PA	9	4.05 ± 0.04	8	4.25 ± 0 08	8	4.03 ± 0.05	8	3.98 ± 0.10
	Br_2FA	13	3 95 ± 0.05	8	3.95 ± 0.05	8	3.92 ± 0.04	8	3.86 ± 0.05
	YP-279	11	3.50 ± 0.03	8	3.61 ± 0.06	8	3.60 ± 0.05	8	3.51 ± 0.03
	YP-614	15	3.41 ± 0.01	8	3.43 ± 0.03	8	3.41 ± 0 02	8	3.44 ± 0.03

FA = 5-butylpicolinic acid; 5-pentyl PA = 5-pentylpicolinic acid; Br_2FA = 5-(3′,4′-dibromobutyl)picolinic acid; YP-279 = 5-(*N*,*N*-dimethylthiocarbamoylmethyl)picolinic acid; YP-614 = 5-(*N*,*N*-dimethylthiocarbamoylthiomethyl)picolinic acid monethylamide.

* Percentage relaxation produced by 1×10^{-3} M of each compound. (see Table 1 and Figure 1).

† The ED_{50} value was obtained visually from a plot of percentage relaxation *v.* log concentration of a compound and expressed as the negative logarithm (−log molar ED_{50}). The maximum relaxation induced by the compound was taken as 100%.

‡ Propranolol, atropine or aminophylline was added to the bath 10 min before prostaglandin $F_{2\alpha}$.

n indicates the number of preparations used. Data are expressed as mean ± s.e. mean.

tensive effect in hypotensive patients (Terasawa & Kameyama, 1971) and spontaneously hypertensive rats (SHR) (Hidaka & Takeya, 1972) and a hypotensive action in normotensive man (Hidaka *et al.*, 1973) and animals (Hidaka & Takeya, 1972). These anti-hypertensive and hypotensive actions of FA had been tentatively explained as due to a reduction in noradrenaline biosynthesis through the inhibition of DBH. It was at least 2–4 h after giving FA before a significant reduction in the vascular noradrenaline could be observed (Hidaka *et al.*, 1975). Although the antihypertensive and the hypotensive actions of FA were attributed to the inhibition of DBH (Hidaka *et al.*, 1974b), it is unlikely that such a slow decrease in tissue noradrenaline that is produced by this mechanism could account for the rapid hypotensive effect. The rapid decrease in blood pressure after FA is more probably related to its vasodilator action.

In an attempt to determine why the vascular smooth muscle relaxed with 5-alkylpicolinic acids and their derivatives, the effects of several blocking agents such as propranolol, atropine, aminophylline, haloperidol and ouabain were determined. As shown in Table 2, the vascular relaxations induced by 5-alkylpicolinic acids were not affected by pretreatment with propranolol or atropine. Aminophylline, an adenosine antagonist, did not affect vascular relaxations induced by the 5-alkylpicolinc acids. These results indicate that the arterial relaxations by 5-alkylpicolinic acids were not mediated through β-adrenoceptors or muscarinic receptors. However the vascular relaxations caused by 5-alkylpicolinic acid were enhanced by pretreatment with haloperidol and were attenuated by pretreatment with ouabain. In view of the fact that ouabain attenuated the relaxations produced by 5-alkylpicolinic acids, activation of the electrogenic Na^+ pump has to be considered as a possible mechanism of action. Haloperidol-induced enhancement and

Table 3 Effects of haloperidol and ouabain on various vasodilator agents

Compounds			*Control*		*Haloperidol§ (1 × 10^{-6} M)*		*Ouabain§ (2 × 10^{-7} M)*		*(1 × 10^{-6} M)*
		n		n		n		n	
% Relaxation*	FA	19	60.1 ± 2.6	7	79.3 ± 5.0**	10	49.2 ± 5.4*	9	32.1 ± 5.9***
	5-pentyl PA	9	62.1 ± 3.4	8	83.3 ± 2.5***	6	43.2 ± 7.0*	5	32.1 ± 6.3***
	Br_2FA	13	73.2 ± 1.4	8	87.1 ± 2.5***	7	63.0 ± 3 9*	6	32.4 ± 5.2***
	YP-279	11	64.5 ± 2.6	8	80.4 ± 3.0***	11	54.6 ± 4.7*	6	17.4 ± 4.5***
	YP-614	15	43.1 ± 3.8	8	71.4 ± 3.1***	6	45.3 ± 1 6	6	23.8 ± 4.6*
	Isoprenaline‡	12	18.1 ± 1.6	13	25.2 ± 2.5*			12	7.6 ± 2.4**
	Dibutyryl-cyclic AMP	5	62.6 ± 2.8	5	78.2 ± 3.7*			5	24.8 ± 8.7**
	Aminophylline	11	41.2 ± 3.0	6	55.9 ± 2.9*			5	12.1 ± 3.0***
−Log molar ED_{50}†	FA	19	3.67 ± 0.04	7	3.84 ± 0.05	10	3.83 ± 0.05	9	3.64 ± 0.05
	5-pentyl PA	9	4.07 ± 0.04	8	4.07 ± 0.11	6	3.94 ± 0.10	5	3.82 ± 0.12
	Br_2FA	13	3.87 ± 0.05	8	4.03 ± 0.07	7	3.80 ± 0.06	6	3 55 ± 0.07
	YP-279	11	3.43 ± 0.03	8	3.61 ± 0.05	11	3.59 ± 0.04	6	3.40 ± 0.03
	YP-614	15	3.33 ± 0.01	8	3.45 ± 0.03	6	3.55 ± 0.08	6	3.36 ± 0.03
	Isoprenaline‡	12	6.21 ± 0.13	13	5.91 ± 0.10			12	6.28 ± 0.09
	Dibutyryl-cyclic-AMP	5	3.48 ± 0.06	5	3.49 ± 0.08			5	3.51 ± 0.11
	Aminophylline	11	3.46 ± 0.08	6	3.47 ± 0.07			5	3.47 ± 0.09

Abbreviations as in Table 2.

* Percentage relaxation produced by 1 × 10^{-3} M of each compound (with exception of isoprenaline (1 × 10^{-5} M)) and calculated as in Table 1.

† The ED_{50} value was obtained visually from a plot of percentage relaxation *v.* log concentration of a compound and expressed as the negative logarithm (−log molar ED_{50}). The maximum relaxation induced by the compound was taken as 100%.

‡ Preparation was exposed to phenoxybenzamine (5 × 10^{-6} M) for 60 min and then washed out with drug-free bathing solution before addition of isoprenaline (1 × 10^{-5} M).

§ Haloperidol and ouabain were added 10 min before the addition of prostaglandin $F_{2\alpha}$

n indicates the number of preparations used. Data are expressed as mean ± s.e. mean.

ouabain-induced attenuation vascular relaxation are not specific for 5-alkylpicolinic acids as they similarly affect other vasodilators such as isoprenaline, dibutyryl cyclic AMP and aminophylline. The mechanism of the effects of haloperidol and ouabain on vascular relaxation induced by 5-alkylpicolinic acids is now under investigation.

Our thanks are due to M. Ohara, Kyoto University, for assistance with the manuscript.

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(Received February 4, 1977.
Revised March 20, 1977.)

Br. J. Pharmac. (1977), **61**, 271–278

OPIATE BINDING AND EFFECT IN ILEUM PREPARATIONS FROM NORMAL AND MORPHINE PRETREATED GUINEA-PIGS

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1 Dose-response curves for normorphine in the absence and presence of naloxone have been obtained from myenteric plexus-longitudinal muscle strip preparations from normal and morphine pretreated guinea-pigs. In addition, the high affinity stereospecific binding of [^{3}H]-etorphine has been measured in homogenates of the same tissue.

2 Higher concentrations of normorphine were required to produce 50% inhibition of the electrically stimulated contractions of strip preparations from morphine pretreated animals. There was also an increase in the slope of linearized dose-response curves in opiate-tolerant preparations. Maximum opiate effect was unchanged, and responses to exogenous acetylcholine were not affected by the pretreatment.

3 There was a slight increase in the apparent equilibrium constant for naloxone after morphine pretreatment.

4 Tolerance to opiate effect was not accompanied by a change in the affinity or number of stereospecific binding sites for [^{3}H]-etorphine. Hill plots of [^{3}H]-etorphine binding in both control and morphine pretreated preparations gave slopes close to unity.

5 Most of these results can be explained by the assumption that in tolerant preparations, a certain fractional opiate receptor occupation threshold must be exceeded before opiate effects become apparent. It is suggested that the tissue adapts toward a threshold equivalent to the mean receptor occupancy attained during the period of opiate drug pretreatment.

Introduction

Opiate drugs inhibit the output of acetylcholine from neurones in the myenteric plexus of guinea-pig ileum (Paton, 1957). The receptors mediating this effect are similar to opiate receptors in the central nervous system with respect to (i) the relative potencies of opiate agonists (Paton, 1957; Cox & Weinstock, 1966; Kosterlitz, Lord & Watt, 1972), (ii) antagonism by appropriately substituted opiate drugs (Cox & Weinstock, 1966; Gyang & Kosterlitz, 1966; Kosterlitz & Watt, 1968) and (iii) stereospecificity, both for agonists and antagonists (Cox & Weinstock, 1966). Pretreatment of the guinea-pig with morphine results in a reduced sensitivity to opiates of the subsequently isolated ileum (Haycock & Rees, 1973; Goldstein & Schulz, 1973; Ward & Takemori, 1976). Recently, a high affinity stereospecific component of the binding of opiate drugs to homogenates of longitudinal muscle-myenteric plexus preparations from guinea-pig ileum has been shown to possess the expected characteristics of opiate receptor binding (Terenius, 1973; Creese & Snyder, 1975). The preparation thus permits an analysis of the relationship between receptor binding and drug effect. In this paper we describe experiments on opiate agonist and antagonist sensitivity, and opiate receptor binding, in ileum preparations from normal and morphine-tolerant guinea-pigs.

Methods

Pretreatment of guinea-pigs

Male Hartley guinea-pigs weighing from 250 to 400 g were used. Control and morphine-treated guinea-pigs were selected at random from the same batch, and were housed in adjacent cages with unlimited access to food and water. Morphine tolerance was induced by the subcutaneous implantation of five morphine pellets (each containing 75 mg morphine base; Goldstein & Schulz, 1973) under light ether anaesthesia, at about 15 h 30 min on day 1. On day 4, the animal was either killed by decapitation at 09 h 30 min (i.e. about 66 h after pellet implantation; designated '3 day morphine treatment') and the ileum removed, or a further five pellets were implanted at a different site, and the animal was not killed until day 6 at 09 h 30 min (i.e. about 114 h after the first morphine implantation;

designated '5 day morphine treatment'). About half of the control guinea-pigs underwent a sham implantation procedure (laparotomy, under light ether anaesthesia). This practice was discontinued since the results from these animals were not different from untreated controls.

Measurement of opiate drug effect

Myenteric plexus—longitudinal muscle strips were set up in 5 ml organ baths in Krebs-bicarbonate buffer composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.20, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.1 and choline chloride 20 μM, mepyramine maleate 125 nM) oxygenated with 95% O_2 and 5% CO_2, at 37°C as described by Goldstein & Schulz (1973). Contractions were induced by field stimulation at 0.1 Hz, 0.25 ms pulse duration at maximal voltage (80 V). Responses were recorded isometrically. After a 1 h period of equilibration, during which the bath fluid was changed frequently, a dose-response curve to the standard opiate normorphine hydrochloride was obtained. The drug was left in contact with the tissue for 3 min before the bath fluid was changed. The next normorphine dose was applied to the tissue 3 min after the second of two further washes given at 3 min intervals (i.e. the interval between normorphine doses was 12 minutes). At least four, and usually five or six normorphine doses, covering the range 15% to 75% of maximal effect, were used to construct each dose-response curve; the doses were not administered in an ascending or descending concentration sequence. On completion of the initial dose-response curve, naloxone hydrochloride (10 nM) was added to the bathing fluid and the tissue equilibrated for 10 min before a further dose-response curve was established in the presence of naloxone.

Linear plots of the dose-response curves were constructed by plotting the logit_{10} transformation of the percentage inhibition of contractile tension [$\log (y/100-y)$ where $y=\%$ inhibition] produced by each normorphine dose against log normorphine concentration (nM). This plot is analogous to the Hill plot for ligand—macromolecular interactions. Normorphine IC_{50} values were read from the plot at $\log (y/100-y)=0.000$, and the slope was recorded. The apparent naloxone equilibrium constant (K_e) was calculated from the Schild equation, essentially as described by Kosterlitz & Watt (1968), $K_e=a/(DR-1)$, where $a=$ molar concentration of antagonist (in these experiments 10 nM) and $DR=$ the ratio of normorphine IC_{50} (nM) in presence of naloxone to the normorphine IC_{50} in the absence of naloxone.

On some strips, acetylcholine dose-response curves were also obtained before the administration of naloxone; 30 s before each acetylcholine dose, the stimulator was turned off and acetylcholine was left in contact with the tissue for 20 s after which time the bath fluid was changed. After 40 s the stimulator was turned on for 2 min, and the cycle was then repeated (i.e. time between successive acetylcholine doses was 3.5 minutes). On each tissue, four or five submaximal contractions were obtained and the maximum response to acetylcholine was determined. The submaximal responses, expressed as the logit_{10} transformation of the percentage of maximal contraction were plotted against log acetylcholine concentration (nM).

Measurement of high affinity stereospecific opiate binding

Myenteric plexus—longitudinal muscle preparations from normal and morphine-treated guinea-pigs were washed in Krebs-Tris buffer (mM: NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.20, Tris-HCl 100 (pH 7.4) and choline chloride, 20 μM, mepyramine maleate, 125 nM) at 0°C for 20 min, then blotted, weighed, and chopped with scissors. The chopped tissue was homogenized with a Tissuemizer (Kunkel, GFR) in 10 ml Krebs-Tris buffer and made up to a final tissue concentration of 0.02 g/ml. The homogenate was centrifuged at 10,000 *g* for 15 min, the supernatant discarded, and the pellet resuspended in the same volume of Krebs-Tris buffer. The centrifugation was repeated and the tissue was finally resuspended by hand homogenization in a glass—glass homogenizer in Krebs-Tris buffer at a tissue concentration of 0.02 g/ml. Aliquots (250 μl) of this homogenate were incubated at 37°C for 30 min with 250 μl Krebs-Tris buffer containing either [^{3}H]-etorphine or levorphanol (final concentration 1 μM) plus [^{3}H]-etorphine. The incubates were cooled on ice for 10 min before bound and free radioactivity were separated by filtration through Whatman GF/C glass fibre filters under vacuum. The filter was washed with 4 × 4 ml of ice cold Krebs-Tris buffer, and transferred to a counting vial where it was shaken for 60 min with 1 ml Soulene (Packard, Ill., USA). Finally 10 ml of Dimilume Scintillation Cocktail (Packard) or Neutralizer Cocktail (RPI Corp., Ill., USA) was added, and radioactivity determined in a Packard TriCarb liquid scintillation spectrometer. Counting efficiency was determined by external standardization. The protein content of each homogenate was measured (Lowry, Rosebrough, Farr & Randall, 1951).

In each experiment, four concentrations of [^{3}H]-etorphine between 0.1 and 3.0 nM were used. At each concentration, four samples were incubated without levorphanol, and four samples with the unlabelled ligand. Stereospecific binding was calculated by subtracting mean bound radioactivity with levorphanol from mean bound radioactivity without levorphanol. For each experiment, Scatchard plots were prepared, and the dissociation constant and maximum binding determined by extrapolation.

Materials

[^{3}H]-etorphine was purchased from Amersham/Searle. Naloxone was a gift from Endo Laboratories; levorphanol and levallorphan from Hoffmann-La Roche; normorphine from Merck, Sharpe and Dohme. The pituitary endorphin sample was provided by Dr Susan Gentleman.

Results

Normorphine and naloxone effects in normal and morphine-treated ileum

The strip preparations were washed frequently immediately after being mounted since strips from morphine-treated animals showed a rapidly increasing sensitivity to normorphine during this period, a phenomenon also observed by Schulz & Herz (1976). About 1 h after mounting in the organ bath, consistent responses to normorphine could be obtained in both control and pretreated tissues. Normorphine IC_{50} was 235 nM in untreated strips; after the 3 day morphine treatment this value was increased to 679 nM (Table 1). There was no indication that the maximum response to opiates was reduced by morphine pretreatment; inhibitions of greater than 90% were recorded from both control and morphine treated strips.

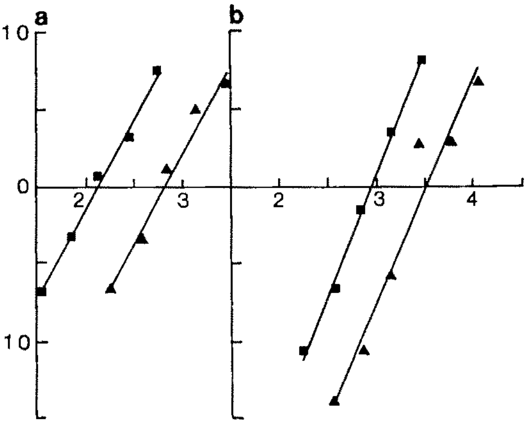

Figure 1 Normorphine concentration-response curves before and after exposure to naloxone. Abscissa scale: log normorphine concentration (nM). Ordinate scale: $logit_{10}$ response (i.e. $log_{10}(y/100-y)$, where $y = \%$ inhibition of contraction). (■) Normorphine responses without naloxone; (▲) normorphine responses in the presence of naloxone (10 nM). (a) Control strip; normorphine $IC_{50} = 135$ nM, naloxone $K_e = 2.68$, mean slope = 1.15. (b) Three day morphine-treated strip; normorphine $IC_{50} = 881$ nM, naloxone $K_e = 3.57$, mean slope = 1.51.

Naloxone antagonized normorphine resulting in a parallel shift of the normorphine dose-response curve to the right in both control and pretreated preparations. Figure 1 shows log normorphine concentration-$logit_{10}$ response plots for typical control and morphine-treated preparations before and after exposure to naloxone. A small increase in the apparent naloxone equilibrium constant as measured by the shift of the normorphine concentration-response curve was consistently observed following morphine treatment (Table 1). There was no evidence of the increase in sensitivity to the antagonist effects of naloxone that is seen in intact animals after opiate pretreatment (Tulunay & Takemori, 1974). The low concentration of naloxone used in these experiments did not induce the sustained contractures that have been reported to occur when the antagonist was applied to ileum preparations that had recently been removed from guinea-pigs given high doses of opiates

Table 1 Normorphine and naloxone activity on ileum from normal and morphine-treated guinea-pigs

Treatment	*Normorphine IC_{50} (nM)*	*Apparent naloxone K_e (nM)*	*Mean slope of log concentration-$logit_{10}$ response curve*
Controls (20)	235 ± 22	2.17 ± 0.12	1.16 ± 0.04†
Morphine, 3 days (28)	679 ± 65*	4.08 ± 0.39*	1.49 ± 0.06*
Morphine, 5 days (12)	428 ± 45*	3.11 ± 0.23*	1.45 ± 0.06*

Figures in parentheses indicate the number of preparations in each treatment group. Normorphine IC_{50} values (±s.e. mean) were determined as described in Methods for each tissue. The apparent naloxone K_e (±s.e. mean) was computed (see: Methods) from the shift of the normorphine concentration-response curve in the presence of naloxone (10 nM). The slope of the normorphine log concentration-$logit_{10}$ response curve for each preparation was determined both before and during exposure to naloxone. Naloxone did not significantly alter the slope, the Table therefore presents mean values (±s.e. mean). Morphine treatments are described in Methods.

* Significantly different from control values ($P < 0.01$); † not significantly different from 1.00 ($P > 0.05$).

(Ehrenpreis, Greenberg & Comarty, 1975; Schulz & Herz, 1976).

The slope of the linearized normorphine dose-response curve was increased following morphine pretreatment (Table 1, and Figure 1). This increase in slope was evident not only in the transformed response measurements, but also by visual inspection of the effects of normorphine doses on the strip preparations. Furthermore, the increase in slope was also apparent after antagonism of the opiate effect by naloxone (Figure 1). Increasing the duration of prior exposure to morphine did not result in a further increase in tolerance to normorphine, but the changes observed in preparations from 3 day morphine-treated animals were also seen after 5 day morphine treatment (Table 1).

Cross tolerance to pituitary endorphin

In a second series of experiments concentration-response curves for normorphine and a pituitary endorphin preparation were compared in strips from control and morphine-treated animals (Table 2). Exposure to morphine resulted in a lower sensitivity to both normorphine and the pituitary extract, and in each case there was a significant increase in the slope of the log concentration-logit_{10} response curves.

Effects of acetylcholine on normal and morphine-treated ileum

The concentrations of acetylcholine giving 50% maximal contractions, and the slopes of the log concentration—logit_{10} response plots for acetylcholine are presented in Table 3. Clearly the effects of acetylcholine on the preparations were not affected by morphine pretreatment of the guinea-pigs. Similar results have previously been reported (Goldstein & Schulz, 1973).

High affinity stereospecific opiate binding

At least two classes of opiate binding sites can be discerned in preparations of guinea-pig ileum (Creese & Snyder, 1975; Cox, Opheim & Goldstein, 1976); in the present experiments, drug concentrations have been selected so that the measured binding is predominantly to the high affinity sites that have been

Table 2 Effects of a pituitary endorphin on ileum preparations from normal and morphine-treated guinea-pigs

Drug and function	*(a)* *Control strips*	*(b)* *3 day morphine strips*	*Ratio (b)/(a)*
Normorphine IC_{50} (nM)	172±40	459±54	2.67
Slope	1.10±0.07	1.65±0.07	1.50
n	12	10	–
Pituitary endorphin			
IC_{50} (µl)	3.82±1.12	8.05±0.89	2.11
Slope	0.96±0.07	1.52±0.17	1.59
n	5	6	–

Results are means ± s.e. mean. IC_{50} values and slopes were obtained from log concentration-logit_{10} response plots. *n* = number of preparations in each group. Preparation of the partially purified pituitary endorphin sample is described elsewhere (Gentleman, Ross, Lowney, Cox & Goldstein, 1976). The morphine treatment is described in Methods.

Table 3 Effects of acetylcholine on normal and morphine-treated ileum

Treatment	*Acetylcholine concentration giving 50% of maximum effect (nM)*	*Slope of log conc-logit_{10} response curve*
Controls (8)	53±15	1.14±0.09
3 day morphine (8)	64±11	0.98±0.05
5 day morphine (12)	60±14	1.02±0.10

Results are mean values ± s.e. mean. The number of preparations in each treatment group is indicated in parentheses. Acetylcholine concentrations giving 50% of the maximum contraction were read from the log concentration-logit_{10} response curve. Differences between the treatments are not significant (*t* test; $P > 0.05$). Morphine treatments are described in Methods.

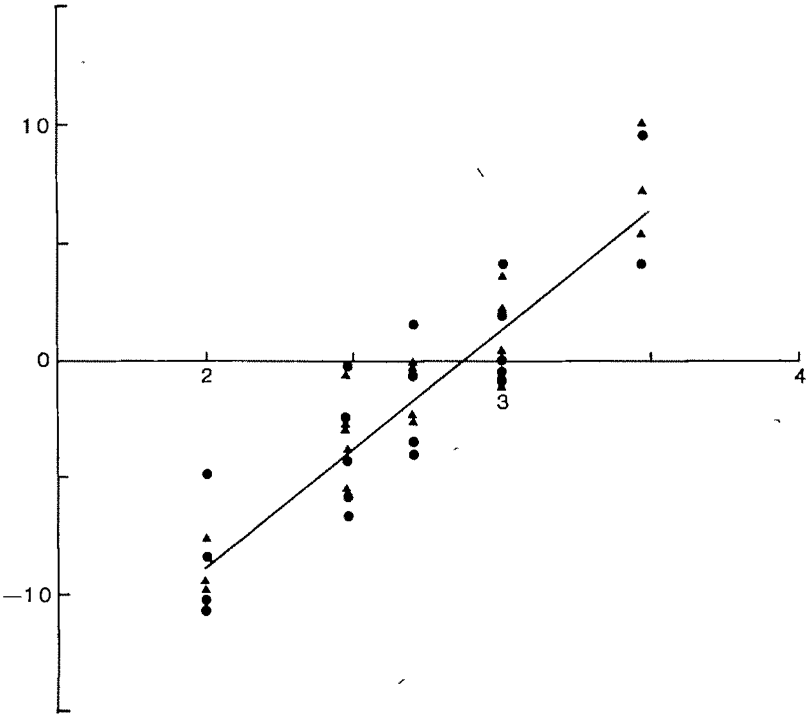

Figure 2 Hill plot of [^{3}H]-etorphine binding by ileal preparation homogenates; accumulated data from experiments shown in Table 4. Abscissa scale: log concentration (pM) of [^{3}H]-etorphine. Ordinate scale: $\log_{10}(Y/1-Y)$, where Y=stereospecifically bound [^{3}H]-etorphine expressed as a fraction of the maximum binding capacity calculated for each each experiment by extrapolation of the Scatchard plot. (●) Control preparations; (▲) 3 day morphine preparations. The line of best fit for the 3 day morphine preparations is drawn; slope=1.01. (Control slope=1.09.)

shown to be stereospecific. The tissues were extensively washed before use to ensure removal of residual morphine in pretreated preparations. The stereospecific binding of [^{3}H]-etorphine to ileum homogenates from control and morphine pretreated guinea-pigs was measured. Dissociation constants and binding capacity were estimated from Scatchard plots for each experiment (Table 4). Neither the dissociation constants nor the number of binding sites were changed by pretreatment with morphine. A Hill plot was constructed for the accumulated [^{3}H]-etorphine binding data (Figure 2). From this plot the mean etorphine dissociation constant was estimated at 0.68 nM in control preparations and 0.73 nM in preparations from 3 day morphine-treated animals. The slopes of the Hill plot for homogenates from

Table 4 [^{3}H]-etorphine binding to homogenates of ileum preparations from normal and morphine-treated guinea-pigs

Treatments	*[^{3}H]-etorphine K_D (nM)*	*[^{3}H]-etorphine binding capacity (fmol/5 mg tissue)*
Controls (6)	0.65±0.08	14.2±3.1
Morphine, 3 days (6)	0.79±0.16	11.7±1.6

Figures are mean values±s.e. mean. The morphine treatment is described in Methods. The figures in parentheses indicate the number of separate experiments. K_D values, and the capacity of high affinity binding sites were computed from Scatchard plots of [^{3}H]-etorphine binding to homogenates of ileum at concentration between 0.1 and 3.0 nM, in each experiment. Differences between morphine-treated and control groups were not statistically significant (t test; $P > 0.05$).

untreated animals was 1.09, and for homogenates from 3 day morphine-treated animals was 1.01.

The quantity of opiate drug bound has not been related to the protein content of the homogenate in these experiments since it was found that the protein content of strips from 3 day morphine-treated guinea-pigs (100 ± 8 µg protein as bovine serum albumin/5 mg tissue wet weight; mean ± s.e. mean, $n=6$) was significantly higher ($P<0.05$) than control strips, 66 ± 8 µg/5 mg, $n=6$), despite the fact that the morphine-treated animals lost about 18% of their total body weight following implantation. Only a small proportion of the tissue in the strip preparations is neuronal, and hence this increase in protein concentrations probably reflects an adaptive change in the smooth muscle; its relevance to opiate binding and effect is unclear.

Discussion

Our experiments confirm that pretreatment of guinea-pigs with morphine results in a reduction in the sensitivity of subsequently isolated ileal preparations to opiate drugs (Haycock & Rees, 1971; Goldstein & Schulz, 1973; Ward & Takemori, 1976). Cross tolerance to pituitary endorphin was also observed; Waterfield, Hughes & Kosterlitz (1976) have recently reported cross tolerance between morphine and methionine-enkephalin in this preparation. Schulz & Herz (1976) have shown that ileal strips display very great insensitivity to normorphine immediately after removal from the morphine-treated animals, or after being maintained in an opiate containing medium, but that extensive washing reduces that insensitivity. This paper is concerned with strips and homogenates of ileum that were repeatedly washed before use; normorphine responses were consistent over a period of several hours, and the IC_{50} values of tolerant strips were similar to those reported by Schulz & Herz (1976) for extensively washed preparations. It is possible therefore that there are two processes with differing decay times underlying the opiate tolerance seen in isolated ileum strips; a biphasic recovery pattern has also been observed when tolerance to the analgesic effects of opiates has been measured in intact rats (Cox, Ginsburg & Willis, 1975). We discuss here only the slowly decaying phenomena.

The etorphine dissociation constant measured in Krebs-Tris buffer with homogenates of ileum preparation (Table 1) was similar to the etorphine dissociation constant measured in intact strip preparations in Krebs-bicarbonate buffer (Cox *et al.*, 1976), where the estimated etorphine dissociation constant (0.4 nM) and the etorphine concentration giving 50% inhibition of the electrically stimulated contractions of the ileum preparation (0.5 nM) were shown to be similar. It was also shown in these experiments and by Creese & Snyder (1975) that the high affinity component of opiate binding was stereospecific. Thus these binding sites display the expected properties of receptors mediating the characteristic opiate effects on guinea-pig ileum preparations. Furthermore, the data suggest that there are no spare receptors (Stephenson, 1956) in this system (Creese & Snyder, 1975).

The three day morphine pretreatment resulted in a 2.5 to 3-fold increase in the normorphine IC_{50}. However, there was no change in measured opiate agonist binding affinity for the receptors. On the basis of the variances seen in these experiments even a two-fold increase in K_D would have been readily detected. There was also no change in the number of high affinity binding sites following morphine pretreatment. Hill plot slopes of close to unity indicate that there was neither positive nor negative cooperativity of binding in control or morphine treated preparations. The failure of opiate pretreatment to alter binding characteristics of opiate receptors in rat brain (Klee & Streaty, 1974) and in neuroblastoma X glioma hybrid cells in culture (Sharma, Klee & Nirenberg, 1975) has previously been described.

In control strips the log normorphine concentration-$logit_{10}$ response curve had a slope that was not significantly different from unity. This plot is analogous to the Hill plot for receptor-ligand interactions. Since the events linking receptor occupation and inhibition of acetylcholine release are unknown, a slope of unity may be coincidental. Nevertheless, the data are consistent with the hypothesis that opiates inhibit a rate-limiting step in processes coupling electrical stimulation to transmitter release, the magnitude of opiate effect in control preparations being determined solely by simple mass action equilibria at the opiate receptor. A more complex situation must exist in the tolerant state since the slope of the linearized concentration-response curve increased to 1.5 following morphine pretreatment.

Some postulated mechanisms for tolerance development are inconsistent with the phenomena we have observed. Thus there is no supersensitivity to the relevant neurotransmitter, acetylcholine (Goldstein & Schulz, 1973), as proposed by Collier (1966). There is no increase in the number of 'silent receptors' for opiates (Collier, 1966), or in the number of molecules of an enzyme directly inhibited by opiate drug (Goldstein & Goldstein, 1968); both of these hypotheses require that there should be an increase in the number of opiate binding sites. It is possible that the general principle of de-repression of enzyme synthesis following drug-induced inhibition (Goldstein & Goldstein, 1961, 1968) might be applied to enzymes indirectly inhibited by opiates, as proposed by Sharma *et al.* (1975). However this type of model, without modification, does not appear to predict an increase in the slope of the linearized concentration-response curve. One possible interpretation of the increase in slope arises from theoretical formulations by Goldstein, Aronow & Kalman (1974). If the opiate inhibited process loses its rate-limiting property as a result of morphine pretreatment, then no opiate effect would be observed until a certain fractional receptor occupancy had been exceeded, although the maximal response (complete inhibition) would be unchanged. A consequence of the existence of a receptor occupancy threshold is that the concentration-response curve is steeper than predicted by simple mass action equilibria (Goldstein *et al.*, 1974). Thus tolerance would result, and the concentration-response curve would increase in slope, if the system adapted to the continued presence of opiate by introducing an opiate receptor occupancy threshold. An adaptive response of this type offers the potential advantage that during chronic drug administration, exogenous opiate occupies a proportion of the receptors corresponding to the threshold, allowing endogenous opioid peptides to continue to exert some control on the transmitter release process by increasing receptor occupancy above threshold.

In tolerant strips the antagonistic effects of naloxone were slightly reduced. A similar reduction of naloxone effect in ileum preparations from morphine pretreated guinea-pigs has been observed by Ward & Takemori (1976), although the absolute values of estimated equilibrium constants (as calculated from the pA_2 values they report) are four to six-fold higher than we have found. (The control naloxone K_e value in our experiments is close to that found by Kosterlitz & Watt, 1968). The significance of this reduction of naloxone sensitivity is uncertain. *In vivo* pretreatment with opiates results in an increase in the ability of naloxone to antagonize opiate-induced analgesia (Tulunay & Takemori, 1974). However, recent work has suggested that this increase in naloxone sensitivity is not necessarily associated with, and may be unrelated to, the tolerant state (Harris, Loh & Way, 1976).

Levorphanol tartrate was given by Hoffman La-Roche, normorphine hydrochloride by Merck, Sharpe and Dohme, and naloxone hydrochloride by Endo Laboratories. We thank Dr A. Goldstein for helpful discussion and advice on the preparation of the manuscript. This work was supported by National Institute on Drug Abuse grants 972 and 1199.

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THE EFFECT OF ATROPINE ON ACID SECRETION STIMULATED BY ACETYLCHOLINE, HISTAMINE AND GASTRIN IN THE ISOLATED WHOLE STOMACH OF THE RAT

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1 An isolated stomach preparation from immature rats has been used to study the effect of atropine on gastric acid secretion.

2 The acid secretory response to acetylcholine was not inhibited by atropine at a concentration of 0.3 μM. Concentrations of atropine of 1 to 3 μM produced a measurable inhibition of acid secretion, and a concentration of atropine of 10 μM caused a complete block of acid secretion which could not be surmounted by high concentrations of acetylcholine.

3 The acid secretory response to histamine was not inhibited by concentrations of atropine of up to 1 mM.

4 Concentrations of atropine of 1 μM and 10 μM did not inhibit gastrin-stimulated acid secretion, although a significant inhibition of acid output was observed with atropine at concentrations of 0.1 mM and 1 mM.

5 These findings are discussed in relation to the role of cholinergic mechanisms in the control of gastric acid secretion.

Introduction

Cholinergic activity exerts a considerable influence on gastric acid secretion through the direct stimulation of parietal cells, the release of gastrin from the pyloric antrum, and by modifying the responsiveness of the parietal cells to gastrin and histamine. These cholinergic influences are controlled to some degree by long vago-vagal reflexes (Harper, Kidd & Scratcherd, 1959; Grossman, 1962; Debas, Konturek, Walsh & Grossman, 1974; Debas, Walsh & Grossman, 1975), and by local cholinergic reflexes which have been identified in the absence of vagal innervation (Grossman, 1961; Magee & Hu, 1975). A possible role for a local cholinergic mechanism in the control of acid secretion has been studied using atropine in animals in which the vagal innervation to the whole stomach or a fundic pouch has been severed, although it must be appreciated that atropine may also influence acid secretion by inhibiting gastrin release from the pyloric antrum; an effect which remains to be firmly established (Smith, Kewenter, Connell, Ardill, Hayes & Buchanan, 1975). In addition, gastrin has been shown to release acetylcholine from the myenteric plexus in guinea-pig ileum (Vizi, Bertaccini, Impicciatore & Knoll, 1972, 1973) and it is possible that the secretagogue action of gastrin on the stomach is partly mediated through the release of acetylcholine.

Atropine inhibits pentagastrin–and to a lesser extent histamine-stimulated acid secretion in the Heidenhain pouch dog (Code, Hightower & Hallenbeck, 1951; Janowitz & Hollander, 1956; Grossman & Konturek, 1974), pentagastrin-stimulated acid secretion in the Heidenhain pouch rat (Johansson, Lundell & Svensson, 1971), pentagastrin-stimulated acid secretion in the Heidenhain pouch cat (Svensson & Emås, 1974) and both pentagastrin- and histamine-stimulated acid secretion in vagotomized conscious cats provided with a gastric fistula (Emås, 1968). Konturek, Oleksy & Wysocki (1968) have also reported that atropine produced a slight inhibition of acid secretion stimulated by histamine or pentagastrin, in duodenal ulcer patients subjected to vagotomy and pyloroplasty.

The effect of atropine on acid secretion in isolated stomach preparations has not been extensively studied, although high concentrations of atropine (1–10 mM) have been used to inhibit histamine- and pentagastrin-stimulated acid secretion in isolated gastric mucosa preparations of amphibians (Thorpe & Durbin, 1969, 1972; Nakajima, Shoemaker, Hirschowitz & Sachs, 1970). In the present work the effect of atropine on acid secretion stimulated by histamine, gastrin and acetylcholine has been investigated in the isolated whole stomach of the rat in which both neural and hormonal influences have been minimized.

Methods

Gastric acid secretion in the isolated stomach of the immature rat (35–45 g) was measured by the methods described previously by Bunce & Parsons (1976) and Bunce, Parsons & Rollings (1976). In brief, the rats were anaesthetized with pentobarbitone, the stomach exteriorized and the oesophagus ligated. An incision was made in the rumen of the stomach, and the contents washed out with warm Krebs-Henseleit solution. A second incision was made at the pyloric sphincter and polythene cannulae were inserted and tied into the stomach via these incisions. The stomach was rapidly dissected out and placed in Krebs-Henseleit solution at 37°C.

The lumen of the stomach was perfused at a rate of 1 ml/min with a modified Krebs-Henseleit solution from which the buffers ($NaHCO_3$ and KH_2PO_4) were omitted. The hydrogen ion concentration of the effluent perfusate from the stomach was continuously recorded, and the rate of acid secretion expressed as nmol per minute. After setting up the stomach preparation the basal H^+ output was allowed to stabilize, under control conditions and in the presence of atropine, before the secretory responses to an agonist were investigated. The response to a single dose of an agonist was calculated as the amount of acid secreted at peak response minus the preceding basal level. Fresh solutions of histamine, acetylcholine and atropine were made up each day in Krebs-Henseleit solution. A stock solution of gastrin at 2.5 mg/ml was prepared in 0.05 M NH_4HCO_3. This solution was stored at −5°C and diluted with Krebs-Henseleit solution as required.

Drugs

The following drugs were used: histamine acid phosphate, acetylcholine chloride and atropine sulphate (BDH Ltd.), gastrin (synthetic human gastrin I, Research Plus Laboratories Inc.), pentobarbitone (Sagatal, May & Baker Ltd.).

Analysis of results

Results are expressed as mean ± s.e. mean. An analysis of variance was used to test the effect of atropine on acid secretion, and potency ratios were calculated according to Finney (1964). Dose-ratio (DR) is the reciprocal of potency ratio. A P value of less than 0.05 was considered to be significant.

Results

The design of the present experiments was essentially the same as for those described previously in which the effects of metiamide on acid secretion were investigated in the same preparation (Bunce & Parsons, 1976; Bunce *et al.*, 1976). Suitable doses of both histamine and acetylcholine were chosen for carrying out 2+2 assays which were used to determine the effects of atropine. With gastrin, two doses of the agonist were used under control conditions followed by three doses of gastrin in the presence of atropine. Since in the present investigation atropine (1 mM) did not inhibit histamine-stimulated acid secretion the appropriate control experiments described by Bunce & Parsons (1976) were not repeated. However, atropine did inhibit acid secretion stimulated by both acetylcholine and gastrin and it was therefore considered necessary to repeat in this study the corresponding control experiments in the absence of an antagonist. As reported previously (Bunce *et al.*, 1976) no time-related changes in the sensitivity of the rat isolated stomach to either gastrin or acetylcholine were observed. As in previous experiments (Bunce *et al.*, 1976) 'priming' doses of gastrin and acetylcholine at 0.1 µM and 0.3 mM respectively were used before constructing dose-response curves. These 'priming' doses were not required when histamine was used as the secretagogue.

Basal acid secretion

The basal level of acid secretion was calculated immediately before an agonist dose was administered both under control conditions and in the presence of atropine. The results are shown in Table 1. Concentrations of atropine up to 10 µM did not produce a significant effect, but concentrations of atropine of 0.1 mM and 1 mM did significantly inhibit basal acid secretion.

Acetylcholine

The dose-response curve to acetylcholine is linear over the range 0.3 mM to 1 mM (Bunce *et al.*, 1976). A control 2-point dose-response curve was established on each stomach in the absence of atropine with concentrations of acetylcholine of 0.3 mM and 0.7 mM. The stomach was then equilibrated in Krebs-Henseleit solution containing the appropriate concentration of atropine for approximately 1 h, and the second curve constructed. Atropine was used at concentrations of 0.3, 1, 2, 3 and 10 µM. Preliminary experiments showed that no inhibition of acetylcholine-stimulated acid secretion was observed in the presence of 0.3 µM atropine. Atropine in the concentration range 1–3 µM produced an inhibition of acid secretion which could

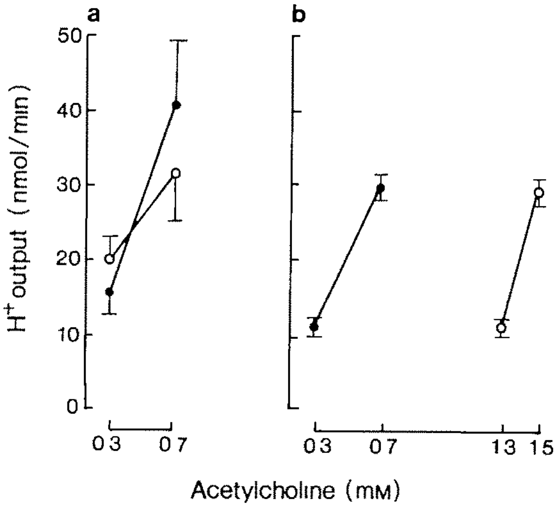

Figure 1 Dose-response curves to acetylcholine in the presence and absence of atropine. Vertical lines show s.e. mean. Analyses of variance on these data provided the following information. (a) Control ($n=8$); the second curve (○) is not significantly displaced from the first curve (●); the slopes of the two lines are not significantly different; (b) 2 µM atropine ($n=8$), the slope of the line in the presence of atropine (○) is not significantly different from that obtained with acetylcholine alone (●); the dose-response curve to acetylcholine in the presence of atropine is significantly displaced from the control curve.

Table 1 The effect of atropine on basal acid secretion in isolated whole stomach of the rat

Acid output (H^+ nmol/min ± s.e. mean)		
Control	*Atropine*	
	1 µM	
30.8 (±3.8, $n=16$)	31.9 (±4.6, $n=16$)	$t=0.19, P>0.05$
	3 µM	
35.1 (±3.9, $n=20$)	26.7 (±2.4, $n=20$)	$t=1.82, P>0.05$
	10 µM	
42.8 (±7.2, $n=10$)	29.5 (±3.5, $n=10$)	$t=1.66, P>0.05$
	0.1 mM	
32.2 (±1.7, $n=12$)	22.9 (±2.3, $n=12$)	$t=3.23, P<0.01$
	1 mM	
31.9 (±3.5, $n=20$)	21.8 (±2.2, $n=20$)	$t=2.43, P<0.05$

be measured, but in the presence of 10 µM atropine concentrations of acetylcholine of 3 mM and 10 mM failed to stimulate acid secretion. The mean dose-response curves obtained under control conditions and in the presence of 2 µM atropine are shown in Figure 1, and details of this result together with those obtained using atropine at concentrations of 1 µM and 3 µM are recorded in Table 2. The dose-ratios obtained with 1 µM and 2 µM atropine were significantly different, but 3 µM atropine did not give a dose-ratio which was significantly greater than that obtained with 2 µM atropine. Although these results (Table 2) show that atropine at high concentrations is an effective inhibitor of acetylcholine-stimulated acid secretion in the isolated stomach, the plot of $\log_{10}$ (DR−1) against $\log_{10}$ [atropine] (Arunlakshana & Schild, 1959) using these data predictably showed a significant deviation from linearity and hence did not provide evidence of competition between acetylcholine and atropine.

Histamine

The histamine dose-response curve is linear over the range 10 µM to 0.1 mM histamine (Bunce & Parsons, 1976), and concentrations of histamine of 30 µM and 0.1 mM were therefore used for the construction of 2-point dose-response curves. The effect of atropine was investigated by the same experimental procedure as described for acetylcholine. Atropine was used at a concentration of 1 mM and the mean result from 8 experiments is shown in Figure 2. High concentrations of atropine (1 mM) failed to inhibit histamine-stimulated acid secretion.

Gastrin

The gastrin dose-response curve is linear over the range 30 nM to 0.3 µM (Bunce *et al.*, 1976), and concentrations of gastrin of 0.1 µM and 0.3 µM were used for the construction of the control 2-point dose-response curves. It has been shown previously (Bunce *et al.*, 1976) that, in the presence of metiamide, gastrin at 0.5 µM gave a smaller acid secretory response than 0.3 µM gastrin, and for this reason in the present experiments the concentration of gastrin was increased to 0.5 µM in the presence of atropine to

Table 2 The effect of atropine on acetylcholine-stimulated acid secretion in isolated whole stomach of the rat

Atropine (µM)	*Dose-ratio (95% confidence limits)*
1	2.7 (1.7–4.2)
2	7.9 (5.2–11.6)
3	8.3 (5.6–12.2)

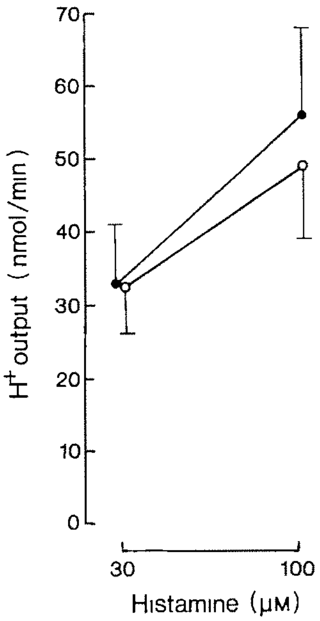

Figure 2 The effect of atropine (1 mM) on the acid secretory response to histamine. Control (●), atropine (○). Each point is the mean of 8 observations. Vertical lines show s.e. mean. An analysis of variance on these data provided the following information. The dose-response curve to histamine in the presence of atropine is not significantly displaced from the control curve; the slopes of the two lines are not significantly different.

determine whether a similar result was obtained. The experimental protocol was as described for acetylcholine.

Concentrations of atropine of 1 μM and 10 μM did not inhibit gastrin-stimulated acid secretion, although a significant inhibition of acid output was observed with atropine at concentrations of 0.1 mM and 1 mM (Figure 3). The results recorded in Figure 3 also show that in the presence of atropine (0.1 mM and 1 mM) the mean acid secretory response to 0.5 μM gastrin is greater than the response to 0.3 μM gastrin (cf. the comparable data obtained in the presence of metiamide by Bunce *et al.* (1976)).

Discussion

The present investigation shows that acetylcholine-stimulated acid secretion is inhibited by concentrations of atropine of 1 to 3 μM. The latter concentrations are relatively high when compared with the concentration of atropine used to block muscarinic receptors ($pA_2 = 8.8$, equivalent to a concentration of 1.6 nM, Arunlakshana & Schild, 1959). Similar results have been obtained by other workers using concentrations of atropine ranging from 0.1 μM to 30 μM to inhibit acid secretion stimulated by cholinomimetic compounds in isolated gastric mucosa preparations of amphibians (Thorpe & Durbin, 1969, 1972; Nakajima *et al.*, 1970; Shoemaker, Makhlouf & Sachs, 1970; Goto & Watanabe, 1975). Arunlakshana & Schild (1959) have reported that a pA_{10} value for atropine of 4.2 (equivalent to a pA_2 value of 5.15) was obtained on the frog rectus which was considered to possess nicotinic receptors. Therefore although bethanechol, a predominantly muscarinic agonist (Koelle, 1975), does stimulate acid secretion in the rat isolated stomach (Bunce & Parsons, unpublished observations) the possibility remains that nicotinic receptors are partly involved in the acid secretory response to acetylcholine. However, dimethylphenylpiperazine (DMPP), an agonist at nicotinic receptors (Chen, Portman & Wickel, 1951), does not stimulate acid secretion in anaesthetized rats (Doss & van Zwieten, 1972) or conscious dogs (Odori & Magee, 1969).

Atropine has previously been shown to be a competitive antagonist at muscarinic receptors (Arunlakshana & Schild, 1959) and evidence was therefore sought for competition against acetylcholine in this preparation. Although atropine produced a parallel shift to the right of the acetylcholine dose-response curve (Figure 1) additional studies provided no evidence to support the supposition that atropine was inhibiting acetylcholine-stimulated acid secretion in a competitive manner. Firstly, the inhibition of acid secretion by 10 μM atropine was not surmounted by concentrations of acetylcholine up to 10 mM. However, concentrations of acetylcholine greater than 1 mM also failed to stimulate acid secretion under control conditions (Bunce *et al.*, 1976), and it is therefore possible that these large concentrations of acetylcholine inhibit acid secretion by a mechanism unrelated to their interaction with cholinergic receptors. Secondly, the plot of $\log_{10}$ (DR−1) against $\log_{10}$ [antagonist] according to the equation for competitive inhibition was not linear. Comparable studies on mammalian isolated stomach or gastric mucosa preparations have not been reported, but it has been suggested by Thorpe & Durbin (1972) that atropine is a competitive inhibitor of acetylcholine in the amphibian isolated gastric mucosa preparation.

The inhibition of gastrin-stimulated acid secretion in the present study only occurred at high concentrations of atropine (0.1 mM and 1 mM) and the result must be interpreted with caution. This inhibition cannot be ascribed to a non-specific effect on the acid secretory mechanism since histamine-stimulated acid secretion was not inhibited by 1 mM atropine, although a non-specific effect at the gastrin receptor cannot be excluded. In the guinea-pig ileum, contraction of the smooth muscle in response to gastrin, which is thought

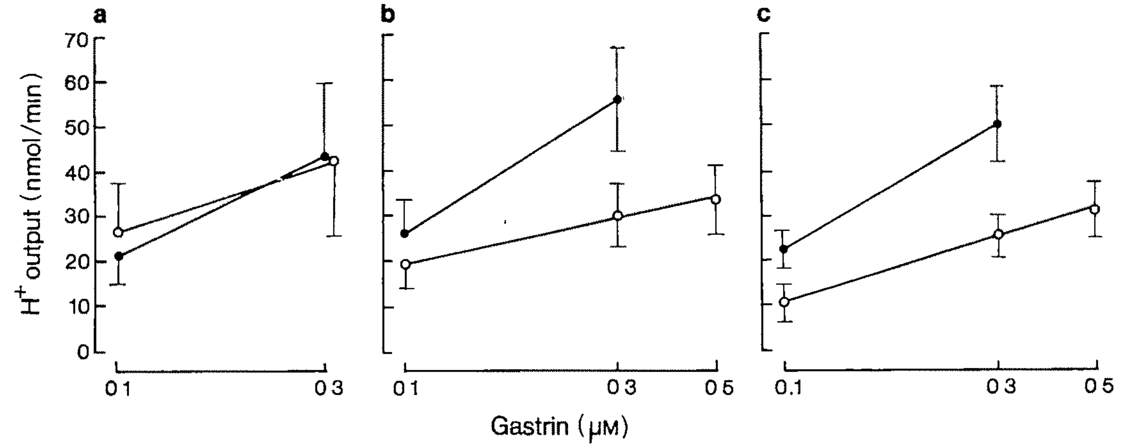

Figure 3 Dose-response curves to gastrin in the presence and absence of atropine. Vertical lines show s.e. mean. Analyses of variance on these data provided the following information. (a) Control ($n=5$); the second curve (○) is not significantly displaced from the first curve (●); the slopes of the two lines are not significantly different; (b) 0.1 mM atropine ($n=6$); (c) 1 mM atropine ($n=10$). In each case the slope of the line in the presence of atropine (○) is not significantly different from that obtained with gastrin alone (●). The dose-response curves to gastrin in the presence of atropine (0.1 mM and 1 mM) are significantly displaced from their respective control curves.

to be mediated via the release of acetylcholine (Vizi *et al.*, 1972, 1973), is inhibited by a dose of hyoscine (15 nM) which also inhibits contractions produced by acetylcholine (Bennett, 1965). Since gastrin-stimulated acid secretion in the isolated stomach of the rat is inhibited by concentrations of atropine (0.1 mM and 1 mM) which are approximately 100-fold greater than those required (1–3 μM) to inhibit acetylcholine-stimulated secretion, it appears unlikely that gastrin is stimulating acid secretion partly through a release of acetylcholine. For comparison, the present work is in agreement with some studies on the amphibian isolated gastric mucosa in which high concentrations of atropine at 2 mM (Thorpe & Durbin, 1972) and 10 mM (Nakajima *et al.*, 1970) were required to inhibit pentagastrin-stimulated acid secretion, although Goto & Watanabe (1975) have reported that tetragastrin-stimulated acid secretion is inhibited by 30 μM atropine in the isolated gastric mucosa of the frog.

Histamine-stimulated acid secretion was not inhibited by high concentrations of atropine (1 mM) in this investigation. This observation indicates that in this preparation a cholinergic mechanism is not involved directly in the acid secretory response to histamine, and the result is also incompatible with the hypothesis of receptor interaction proposed by Grossman & Konturek (1974). Goto & Watanabe (1975) have also reported that atropine failed to inhibit histamine-stimulated acid secretion in amphibian isolated gastric mucosa, although these workers used atropine at a concentration of only 30 μM. In contrast, Thorpe & Durbin (1972) found that 1 mM atropine did inhibit acid secretion in response to histamine in the frog isolated mucosa.

In the present study, doses of atropine which inhibited acetylcholine-stimulated acid secretion (i.e. 1–3 μM) did not affect acid secretion stimulated by either gastrin or histamine, and this result might indicate that the background cholinergic tone in the isolated perfused stomach of the rat, if it exists at all, is very low. This latter assumption conflicts with the observations of Johansson *et al.* (1971) who found that a dose of atropine (100 μg/kg i.v.) which inhibited methacholine-stimulated acid secretion also inhibited gastrin-stimulated acid secretion in the Heidenhain pouch rat. However, Albinus & Sewing (1969) have reported that in the anaesthetized lumen-perfused rat a dose of atropine (0.5 mg/kg i.v.) which inhibits bethanechol-stimulated acid secretion does not affect acid secretion stimulated by either gastrin or histamine. The proposition that the isolated stomach of the rat possesses a low background cholinergic tone would be consistent with the view that the latter mechanism is not responsible for the basal level of acid secretion and with the evidence that the basal acid secretion is not inhibited by atropine. Indeed, inhibition of the spontaneous acid output was only observed in the presence of a high concentration of atropine (0.1 mM–1 mM, Table 1).

We are indebted to Miss P. North and Mr D.L. Daniel for help with the statistical calculations.

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(Received February 25, 1977)

Br. J. Pharmac. (1977), **61**, 285–290

EFFECTS OF 3-METHYLINDOLE IN CATTLE

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1 Rapid intravenous injection of 3-methylindole (3-MI) was shown to induce an anaphylactoid-like reaction in calves.

2 This was suggested by the reduction in response to a repeat dose of 3-MI, by the reduction of effects in the presence of antagonists to the putative mediators of anaphylaxis in cattle and by the production of signs similar to those seen in experimentally induced bovine anaphylaxis.

3 The plasma half-life of 3-MI was short (14.4 min) and, since absorption of 3-MI from the rumen is known to be slow, the extent of formation of 3-MI from L-tryptophan in the rumen would have to be substantial if 3-MI is to be considered the causative agent of 'fog fever', an acute respiratory distress syndrome seen in cattle.

Introduction

Fog fever is an acute respiratory distress syndrome which occurs in adult beef-type cattle soon after a change to better pasture in the autumn (Selman, Wiseman, Breeze, Pirie & Bogan, 1976). The aetiology of fog fever is unknown but it is possible that the condition is the result of an anaphylactic reaction or is caused by the ingestion of a toxic substance in herbage. Most recent investigations have examined the role of pasture constituents, since it has been found that oral doses of the amino acid, L-tryptophan, can give rise to a proliferative alveolitis similar to that seen in fog fever (Dickinson, Spencer & Goreham, 1969). Intravenous doses of L-tryptophan do not induce similar lesions and it is probable that a ruminal metabolite of L-tryptophan is responsible for the lung lesions. The only such metabolite of L-tryptophan which is known to be toxic is 3-methylindole (3-MI; skatole), a compound formed by ruminal microorganisms via indole 3-acetic acid as an intermediate (Carlson, Yokoyama & Dickinson, 1972). A severe proliferative alveolitis occurs 24 to 96 h after oral dosage or slow (6 to 12 h) intravenous infusion of 3-MI (Carlson *et al.*, 1972; Pirie, Breeze, Selman & Wiseman, 1976).

The purpose of this study was to examine the acute effects when 3-MI was given *rapidly* to cattle and to assess whether 3-MI itself had direct pneumotoxic effects, since it was possible that some further metabolite of 3-MI could be involved. The plasma half-life of 3-MI was also measured since the kinetics of 3-MI, i.e. the rate and extent of formation in the rumen, the rate of absorption from the rumen, and the rate of plasma clearance, are important in determining the plasma concentration and thereby the likelihood of 3-MI being the cause of field outbreaks of fog fever.

Methods

Animals

Fourteen parasite-free calves between two and 12 months old were used; the animals were anaesthetized with pentobarbitone (20 mg/kg, i.v.) with additional 2 mg/kg doses intravenously given as required throughout the course of the experiments to maintain anaesthesia.

Intravenous infusions

All previous intravenous administrations of 3-MI have been made using propylene glycol as a solvent (Carlson *et al.*, 1972; Pirie *et al.*, 1976). This solvent is unsuitable for rapid intravenous infusions because of its viscosity so all previous experiments have involved administration over a period longer than 6 hours. Initially we tried ethyl alcohol as a solvent but this was found to be unsatisfactory because 3-MI precipitated out on infusion and also because the alcohol caused venous thrombosis. The solvent chosen was 'Cremophor EL' (Victor Blagdon & Co.), a polyoxyethylated castor oil. This solvent dissolves 3-MI well, is miscible with water and plasma and 3-MI does not precipitate out on infusion. In all experiments 3-MI (Sigma London Chemical Co. Ltd) was administered as a 30 mg/ml solution in 10% 'Cremophor EL' in distilled water.

Infusions were made into the femoral vein via an indwelling cannula and blood sampling for 3-MI determinations was from the other femoral vein. Throughout each experiment, a record was made of carotid arterial pressure, standard limb lead (lead II) ECG, the heart rate from an instantaneous heart rate meter, and respirations via a Fleisch pneumotachograph. Pulmonary arterial pressure was also monitored via an indwelling catheter introduced via the jugular vein; the position of the catheter was verified at necropsy. Arterial pressures were measured with Bell and Howell electronic transducers (Type 4-422-0001) adjusted to the elevation of the anaesthetized animal. All recordings were made on a Devices M19 8-channel pen recorder. Mean arterial pressure was calculated from the formula MAP = Pd + Ps − Pd/3 where Pd = mean diastolic pressure and Ps = mean systolic pressure.

In all experiments the dosage procedure was standardized with intravenous infusion of the dose taking place over 1 min; blood samples for 3-MI determination taken at 2, 5, 10, 20 and 30 min after the end of infusion; infusion of the next dose beginning at 30 minutes.

Drugs

Mepyramine maleate (May & Baker Ltd) (5.0 mg/kg, i.m.), cyproheptadine hydrochloride (Merck, Sharp & Dohme Ltd) (0.3 mg/kg, i.v.) and sodium meclofenamate (Parke, Davis & Co.) (2.0 and 10.0 mg/kg, i.v.) were administered 20 min, 5 min and 5 min respectively before 3-MI administration according to the methods of Aitken & Sanford (1972). Indomethacin was administered intravenously as a 20 mg/ml solution in 95% alcohol 20 min before 3-MI administration.

Measurement of plasma concentrations of 3-methylindole

In six calves (Nos. 3, 4, 5 and 6 (Table 2) and two others, Nos. 13 and 14 in which physiological measurements were not made) plasma levels of 3-MI were measured at times after 3-MI administration until 30 min after the 8 mg/kg dose. The estimation is based on that described by Bradley & Carlson (1974); 1 ml of plasma was shaken for 5 min with 10 ml re-distilled *n*-hexane. The plasma was pipetted off and the hexane layer was concentrated under nitrogen at 50°C to 1 ml and a 5 µl aliquot was injected into the gas chromatograph. 3-MI concentrations were quantified from known standards of 3-MI in *n*-hexane. The chromatographic estimations were made with a Pye 104 gas chromatograph with flame ionization detector. A glass column (1 m × 5 mm) packed with 10% DC 200 on Gas-Chrom Q (100–200 mesh) was used at a column temperature of 150°C and a detector temperature of 250°C. Under these conditions the retention time of 3-MI was about 3 minutes.

Post mortem *and histopathological methods*

Animals 1 and 3 died during the course of the experiment and calves 4 and 7 were killed with pentobarbitone shortly after recovery. These four calves were examined *post mortem* and representative portions of tissue were taken from the lungs, tracheobronchial system and other organs as required. Tissues were fixed in 10% formalin or corrosive formol, dehydrated and double embedded in celloidin and paraffin wax in a vacuum. All sections were cut at 6–8 µm and stained with haematoxylin and eosin.

Results

Response to 'Cremophor EL'

The solvent used (10% 'Cremophor EL' in distilled water) was administered to four calves (Calf numbers 3, 4, 5, 6) during anaesthesia and before administration of 3-MI at a volume equivalent to that used to deliver the highest dose of 3-MI (i.e. 16 mg/kg). No effects were seen on any of the parameters measured.

Response to 3-methylindole

Since doses of 3-MI as high as 60 mg/kg have been infused intravenously over periods of 6–12 h (Carlson, Dickinson, Yokoyama & Bradley, 1975), it was surprising that an initial dose of 4 mg/kg proved fatal in the two calves used; these animals (Nos. 1 and 2) died within 2 min of the end of the injection. By reducing the initial dose it was found that 1 mg/kg was the highest dose that could be given without causing acute deaths.

The response of six calves (Nos. 3 to 8) to an initial 3-MI dose of 1 mg/kg is shown in Table 1. The response varied in each animal but the most consistent finding was pulmonary arterial systolic hypertension, beginning 1 to 3 min after the completion of the infusion, followed by a period of apnoea then a prolonged period of marked tachypnoea and hyperpnoea. Pulmonary arterial diastolic pressure also increased in two of the calves (Nos. 7 and 8) but to a lesser degree than the systolic pressure and in one calf (No. 6) decreased slightly. In all three, however, the mean pulmonary arterial pressure increased.

The marked fall in carotid arterial pressure was not accompanied or followed by a reflex tachycardia. In all cases no change or a slight fall in heart rate was observed with Figures 1 and 2 showing typical responses.

Repetition of the dose of 1 mg/kg 30 min later was

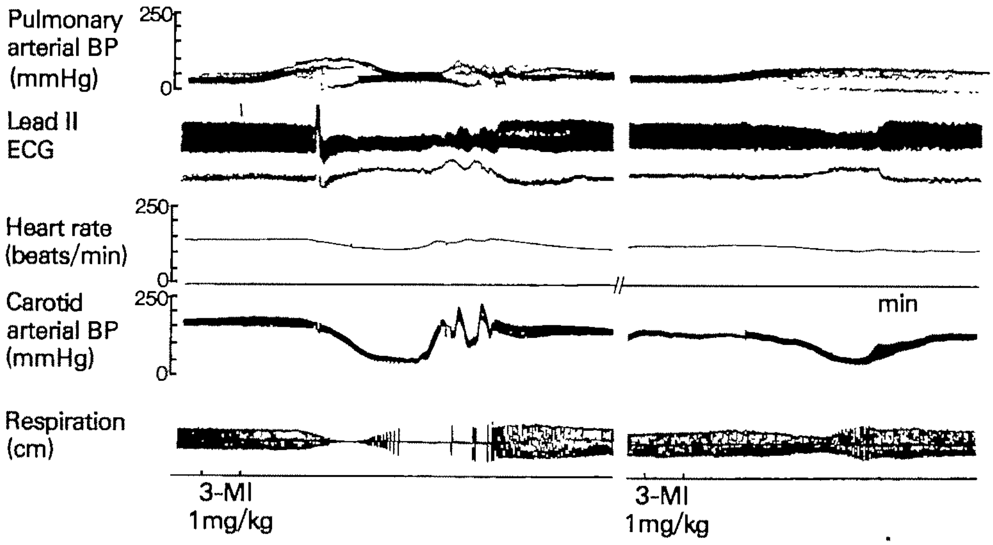

Figure 1 Response of calf (No. 6) to 1 mg/kg 3-methylindole (3-MI) administered intravenously and repeated 30 min later.

accompanied by similar but much reduced responses in all six calves. Typical responses to the initial and repeat doses of 1 mg/kg are shown in Figure 1. Increasing the dose of 3-MI to 2, 4, 8 and 16 mg/kg resulted in similar but more immediate and dose-related effects on respiration which were also accompanied by pulmonary hypertension. Typical responses (Calf No. 4) are shown in Figure 2. The responses of the calves are shown in Table 2.

Effects of antagonists on the response to 3-methylindole

A mixture of antagonists, *viz.* mepyramine, cyproheptadine and indomethacin given to one calf completely eliminated the responses to 3-MI 1 mg/kg. This calf (No. 4) had reacted markedly to this dose of 3-MI one month previously (Table 1).

Two other calves (Nos. 9 and 10) pretreated with

Table 1 Response of calves to an initial dose of 3-methylindole 1 mg/kg and repeated 30 min later

Calf. No.	*3-Methylindole dose (1 mg/kg)*	*Respiratory depth max. decrease (%)*	*Mean carotid arterial blood pressure max. decrease (mmHg)*	*Mean pulmonary arterial blood pressure max. increase (mmHg)*
3	Initial	40	30	ND
	Repeat	0	0	
4	Initial	80	90	ND
	Repeat	0	0	
5	Initial	100(30)*	40	ND
	Repeat	50	0	
6	Initial	100(80)*	110	20
	Repeat	20	70	0
7	Initial	0	0	55
	Repeat	0	0	15
8	Initial	100(40)*	90	30
	Repeat	30	15	10

* = Duration of apnoea in seconds; ND = not determined.

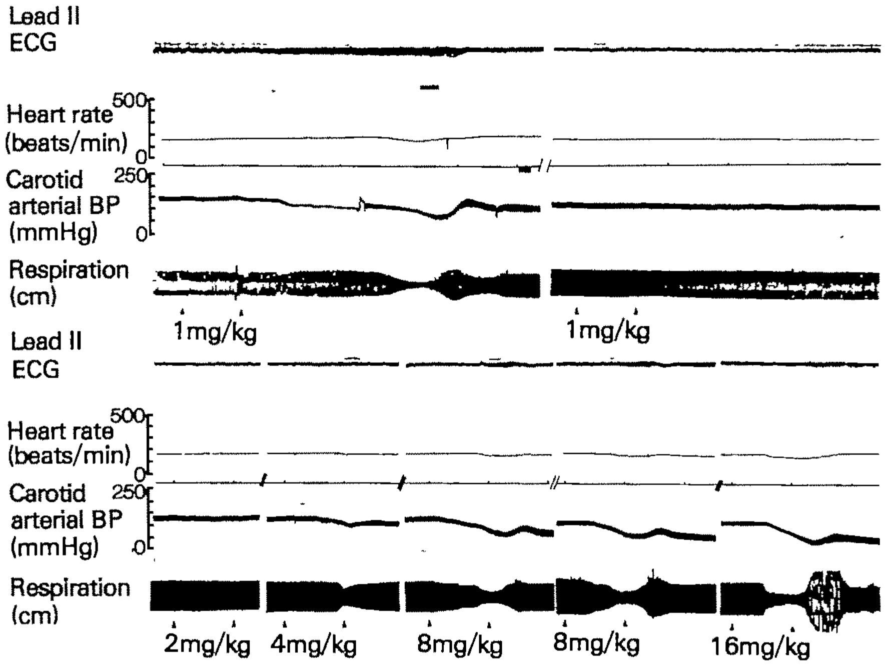

Figure 2 Response of calf (No. 4) to successive doses (1, 1, 2, 4, 8, 8 and 16 mg/kg) of 3-methylindole administered intravenously at 30 min intervals.

Table 2 Responses of calves to successive doses of 3-methylindole given at 30 min intervals

Calf No.	*3-Methylindole dose (mg/kg) 30 min intervals*	*Response*
1	4	Died 1 min post injection
2	4	Died 2 min post injection
3	1,1,2,4,8,16	Died 30 min post 16 mg/kg
4	1,1,2,4,8,8,16	Survived. Respiratory rate elevated for 6 days
4†	1	Survived. No respiratory changes on recovery
5	1,1,2,4,8,16	Died 2 min post 16 mg/kg
6	1,1,2,4,8,16	Survived. Respiratory rate elevated for 7 days
7	1,1	Survived. Respiratory rate elevated for 6 hours
8	1,1	Survived. Respiratory rate elevated for 12 hours
9*	1	Survived. No respiratory changes on recovery
10*	1	Survived. No respiratory changes on recovery
11**	1	Survived. No respiratory changes on recovery
12**	1	Survived. No respiratory changes on recovery

* Pre-treated with sodium meclofenamate (2 mg/kg); **pre-treated with sodium meclofenamate (10 mg/kg); † pre-treated with mepyramine, cyproheptadine and indomethacin.

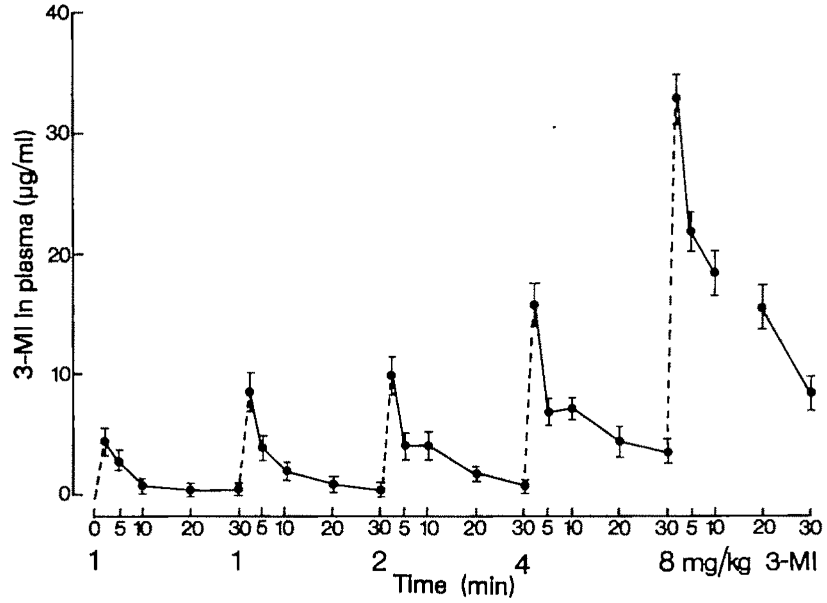

Figure 3 Mean plasma concentrations of 3-methylindole (3-MI) in 6 calves after administration of successive doses (1, 1, 2, 4 and 8 mg/kg) of 3-methylindole intravenously at 30 min intervals. Vertical lines show s.e. means.

sodium meclofenamate (2.0 mg/kg, i.v.) alone showed only a slight reaction to an initial dose of 1 mg/kg 3-MI, respiratory depth being reduced by 30 and 10% respectively with no period of apnoea. In two other calves (Nos. 11 and 12) pretreated with a higher dose of sodium meclofenamate (10 mg/kg, i.v.) no effects on any of the parameters measured were seen at any time after 1 mg/kg 3-MI.

Post mortem *findings*

In calves 1 and 3 (Table 2), which died during the experimental procedure, there was severe pulmonary congestion and oedema. Lung lobules were dark red and fluid ran from their cut surfaces on pressure. Moderate interlobular oedema was also present and white frothy fluid filled the tracheobronchial system. Interstitial emphysema was noted in calf No. 1 and many lung lobules were overinflated in both calves. The congestion and oedema were apparent histologically. Respiratory acini were filled with oedema fluid. Capillaries of the inter-alveolar septa were intensely congested and neutrophils were frequently seen in these vessels. The lamina propria of bronchi and bronchioles was oedematous, particularly in calf No. 3. In this animal, the lung lesions were more severe and additional findings were intra-alveolar haemorrhage, a light exudate of neutrophils and macrophages in the lumina of many small bronchioles and the presence of infrequent globule leukocytes in the basal part of the tracheobronchial epithelium.

Calves 4 and 7 were destroyed by injection of pentobarbitone 8 h and 6 h respectively after the last injection of 3-MI: there were no residual clinical abnormalities at this stage. There was a moderate degree of pulmonary congestion and mild alveolar oedema in scattered acini in both cases. Neutrophils were frequently seen in the capillaries of the interalveolar septa. Animal 7 had lesions of cuffing pneumonia in the cranial lung lobes but the other 3 calves (Nos. 1, 3 and 4) did not have significant lesions of this type.

Plasma half-life of 3-methylindole

The mean plasma concentrations of 3-MI in six calves are shown in Figure 3. After each dose of 3-MI the plasma concentration decreased biphasically with a rapid decline within 5 min and a slower decline from 5 to 30 minutes. Since the initial rapid fall in 3-MI levels is probably a redistribution effect, the mean plasma half-life of 3-MI for doses of 3-MI between 1 and 8 mg/kg (Figure 3) between 5 and 30 min after administration was 14.4 ± 2.5 min (mean ± s.e. mean).

Discussion

The evidence from this work for 3-MI to be considered the causative agent of fog fever in cattle is conflicting.

On the one hand the clinical and pathological signs seen after 3-MI administration are similar to those seen in field cases of fog fever. On the other hand, the kinetics of 3-MI, the slow rate of absorption from the rumen and rapid plasma half-life, would require that formation of 3-MI from L-tryptophan is rapid and extensive.

A small dose of 3-MI administered rapidly intravenously gives rise to an anaphylactoid-like reaction in cattle. This is suggested by (1) production of clinical signs similar to those seen during experimental anaphylaxis in calves, including pulmonary hypertension, systemic hypotension, dyspnoea and apnoea (Aitken & Sanford, 1969); (2) reduced response on repeating the initial dose, presumably due to depletion of mediators, and (3) reduction or abolition of the initial effects by antagonists to known mediators of anaphylaxis in cattle (Eyre, 1971). This reaction may be the result of 3-MI acting as a hapten in an immune reaction or, more directly, by a non-specific mechanism causing release of mediators of anaphylaxis from mast cells, basophils or K-type (Kultschitzky) cells in the pulmonary parenchyma, airways and vasculature. However, it would seem unlikely that 3-MI acts as a hapten, since small amounts of ruminal 3-MI can be demonstrated in healthy cattle, although it is possible that 3-MI is not present in blood in such cases.

From our results, 3-MI given rapidly at doses higher than 1 mg/kg has a more immediate, direct action on bovine lung: the mechanism of this is unknown. Bray, Magnuson & Carlson (1974) have shown that 3-MI intercalates with phospholipid molecules, such as those present in cell membranes and alveolar surfactant. The resultant alterations in cell membrane integrity and alveolar surface tension could result in pulmonary oedema and cell death.

It is interesting that 3-MI has a short plasma half life of about 15 minutes. Carlson *et al.* (1975) have shown that the absorption of oral doses of 3-MI is slow (180 min before peak plasma levels are achieved), so ruminal production of 3-MI from tryptophan would have to be considerable to produce measurable plasma levels of 3-MI. A concentration of approximately 200–300 μg/ml in ruminal fluid would be necessary to maintain a plasma level of 10 μg/ml assuming first-order kinetics for absorption and elimination. It is surprising then, that the levels of 3-MI found in ruminal fluids of cattle dying after being fed L-tryptophan were always low, never rising above 1–10 μg/ml (Yokoyama, Carlson & Dickinson, 1975; Selman *et al.*, 1976). It may be that much higher concentrations are attained in a part of the alimentary tract not sampled. This aspect requires further investigation.

In conclusion, we have shown that 3-MI probably affects the bovine lung directly and not via a metabolite. The immediate clinical signs after rapid 3-MI administration are similar to those seen in experimental anaphylaxis in cattle and this action of 3-MI could well be the result of the release of similar mediators. The half-life of 3-MI in cattle is short (about 15 min) such that marked production of 3-MI from L-tryptophan in the rumen of cattle would be necessary to produce plasma concentrations of the order found after intravenous infusion in these experiments and thus to be considered the causative agent of fog fever.

The Authors wish to thank the ARC for financial support.

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(Received February 28, 1977.
Revised May 5, 1977)

Br. J. Pharmac. (1977), **61**, 291–295

DOPAMINE-INDUCED DEPRESSION OF ADRENERGIC NERVE-MEDIATED CONTRACTION OF SMOOTH MUSCLE

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1 Dopamine (0.25–1.0 μM) applied extraluminally depressed vasoconstrictor responses of the perfused rabbit ear artery to low frequency adrenergic nerve stimulation.

2 The depressant effect of dopamine was prevented in the presence of haloperidol (0.1 μM) but not in the presence of phentolamine (0.03–0.14 μM).

3 Extraluminal dopamine (1 μM) or intraluminal injection of dopamine in amounts up to 0.025 μmol had no dilator effect on arteries partially constricted by infusion of noradrenaline.

4 Dopamine also depressed contractile responses of guinea-pig vas deferens to low frequency adrenergic nerve stimulation. However, in this tissue the effect of dopamine was prevented by phentolamine but not affected by haloperidol.

5 In neither the rabbit ear artery nor the guinea-pig vas deferens did haloperidol increase the magnitude of responses to nerve stimulation or affect muscle sensitivity to noradrenaline.

6 We conclude that the adrenergic axons in the rabbit ear artery, but not the guinea-pig vas deferens, possess specific receptors for dopamine whose activation depresses axonal conduction or transmitter release. However, our results do not favour the view that these receptors are activated during normal transmission at physiological frequencies.

Introduction

It is well documented that α-adrenoceptor agonists depress and α-adrenoceptor antagonists increase the release of noradrenaline from adrenergic axons in response to nerve stimulation (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Starke, 1972 and others), suggesting the existence of presynaptic α-adrenoceptors. From such data it has been postulated that these receptors may be involved in feedback modulation of adrenergic transmission (Farnebo & Hamberger, 1971; Starke, 1971; Kirpekar & Puig, 1971).

Recently it has been reported that low concentrations of dopamine also depress transmitter overflow from the rabbit ear artery (McCulloch, Rand & Story, 1973). This effect of dopamine is prevented by dopamine-receptor antagonists but not by α-adrenoceptor antagonists, suggesting the existence of presynaptic dopamine receptors (Hope, McCulloch, Rand & Story, 1975).

The present experiments have been performed in order to determine the effect of activation of these receptors on neurogenic vasoconstriction in the rabbit ear artery and to obtain evidence as to whether they are involved in normal transmission. Results obtained have been compared with those obtained with the guinea-pig vas deferens.

Methods

Young adult (2.0–2.5 kg) albino rabbits and adult (400–600 g) male guinea-pigs were killed by cervical dislocation. Rabbit ear arteries were prepared as described by de la Lande & Rand (1965) and perfused at a constant rate of 5 ml/min with McEwen's (1956) solution at 37°C. Vasoconstriction was monitored as changes in perfusion pressure via a pressure transducer coupled to the perfusion system distal to the pump.

Vasa deferentia were dissected free of the hypogastric ganglia but without removal of their mesenteric investment and mounted vertically in Huković's (1961) solution at 37°C with a resting longitudinal tension of 250 mg. Longitudinal contractions were monitored via an isotonic strain gauge.

Stimulation of intramural adrenergic nerves was carried out with Pt ring electrodes 10 mm in diameter and 5 mm apart, which were positioned around the proximal end of the artery and the urethral end of the vas deferens. A Grass S44 stimulator was used to deliver trains of 1 ms square wave pulses at 2 pulses/s and 70 volts. With the arteries, the stimulating trains were for 12 s every 200 seconds. With the vasa deferentia, the trains were for 5 s every 80 seconds. The contractile responses obtained were due solely to activation of adrenergic axons and not to activation of the smooth muscle cells, as they were abolished by tetrodotoxin 1.6 μM or guanethidine sulphate 4 μM.

Drugs used were: dopamine hydrochloride (Sigma), haloperidol (Serenace, Searle), noradrenaline hydrochloride (Sigma) and phentolamine mesylate (Regitine, Ciba). Dopamine and noradrenaline were stored as 1 mg/ml solutions in 0.001 N HCl and diluted into 0.9% w/v NaCl solution (saline) on the day of use. All saline used contained 0.6 mM ascorbic acid. Haloperidol and phentolamine were diluted into saline on the day of use from the commercially available ampoules.

Differences of means were assessed using a paired Student's *t* test.

Results

Rabbit ear artery

Nerve stimulation produced increases in perfusion pressure which varied from 20 to 250 mmHg in different preparations. The mean ± s.e. mean for all preparations used was 97 ± 16.3 mmHg ($n = 14$) (1 mmHg = 1.333 mbar).

When dopamine was added to the bathing fluid surrounding the artery to produce a final concentration of 0.25–1.0 μM, the amplitude of responses to nerve stimulation was decreased (Table 1, Figure 1). This depression was rapidly reversed by washing the bath free of dopamine and was reproducible. The maximum depressant effect was observed in the presence of 1.0 μM dopamine. Concentrations greater than this caused direct vasoconstriction which prevented assessment of the amplitude of neurogenic responses.

In order to determine whether dopamine had any depressant effect on the vascular smooth muscle, partial constriction was maintained in three preparations by intraluminal infusion of noradrenaline at a final concentration of 0.5 μM. Under these conditions resting pressure was maintained at a steady level somewhat above 200 mmHg and intraluminal injections of acetylcholine (0.1–0.25 nmol) produced large falls in perfusion pressure, reflecting vasodilatation. In contrast, intraluminal injections of dopamine in doses from 0.025 nmol to 0.025 μmol had no effect on perfusion pressure. Doses of 0.05 μmol produced vasoconstriction. Addition to the extraluminal bathing fluid of 1 μM dopamine similarly had no dilator effect.

In nine experiments the dopamine-receptor antagonist haloperidol was added to the extraluminal fluid in a final concentration of 0.1 μM after reproducible depressant responses of dopamine on nerve-mediated vasoconstriction had been obtained. In the presence of haloperidol the effect of dopamine was greatly reduced (Table 1). This antagonist effect persisted for up to 30 min after washing the bath free of haloperidol. Haloperidol had no appreciable or consistent effect itself on the amplitude of nerve-mediated vasoconstriction or on constrictor responses to intraluminal injections of 0.1 nmol noradrenaline (Table 2).

In six further experiments the depressant effect of dopamine was tested after addition to the extraluminal bathing fluid of phentolamine in final concentrations of 0.03 μM (three experiments) or 0.14 μM (three experiments). These concentrations of phentolamine produced some (up to 30%) reduction in the amplitude of responses to nerve stimulation and somewhat greater reduction (up to 60%) in the amplitude of similar sized responses to extraluminal addition of 0.05–0.25 μM noradrenaline. In contrast

Table 1 Depression by dopamine of adrenergic nerve-mediated contractions of rabbit ear artery and guinea-pig vas deferens and the effect on this of haloperidol and phentolamine. The magnitude of the effect of dopamine is expressed in terms of percentage reduction of control response

			Effect of dopamine		
	Dopamine	*Control*	*Haloperidol*	*Phentolamine*	
Tissue	*(μM)*	*($n = 14$)*	0.1 μM ($n = 9$)	0.03 μM ($n = 5$)	0.14 μM ($n = 3$)
Rabbit ear artery	0.25	30 ± 7%	8 ± 3%*	22 ± 3%	15 ± 4%
	1.0	41 ± 6%	12 ± 5%**	41 ± 4%	38 ± 2%
		Control ($n = 14$)	*Phentolamine* 0.3 μM ($n = 5$)	*Haloperidol* 0.1 μM ($n = 2$)	0.19 μM ($n = 3$)
Guinea-pig vas deferens	1.0	21 ± 5%	2 ± 2%*	15, 30%	42 ± 14%
	5.0	40 ± 6%	20 ± 4%	34, 65%	73 ± 8%

* Different from control values for same tissues ($P < 0.05$); ** different from control value for same tissues ($P < 0.02$).

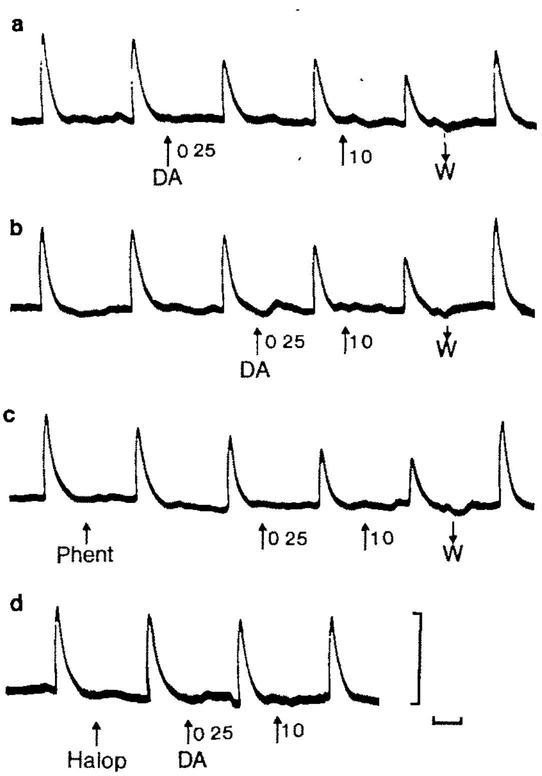

Figure 1 Constrictor responses of a rabbit ear artery perfused with physiological saline at constant rate to stimulation of the periarterial nerve supply (2 Hz, 12 s every 200 seconds). The panels are consecutive and run from above downwards. Addition to the extraluminal bathing fluid of dopamine (DA) at final concentrations of 0.25 μM or 1.0 μM produced dose-dependent depression of the responses which was quickly reversed after the bath was washed free of dopamine (W) and was reproducible. Phentolamine (Phent) 0.14 μM produced slight postsynaptic α-adrenoceptor blockade but did not affect the response to dopamine. By contrast, haloperidol (Halop) 0.1 μM completely prevented the effect of dopamine. Calibrations: 50 mmHg and 1 minute.

phentolamine had no antagonist action towards the depressant effects of either dose of dopamine used (Table 1). Higher concentrations of phentolamine could not be used because they almost completely abolished control responses to nerve stimulation.

Guinea-pig vas deferens

Responses to nerve stimulation ranged from 50–400 mg and had a mean value of 234 ± 30.6 mg ($n = 14$). Addition of dopamine in final concentrations between 1 μM and 5 μM to the bathing fluid produced dose-dependent depression of the amplitude of these responses (Table 1) which was readily reversed by washing the bath and was reproducible. Concentrations of dopamine greater than 5 μM caused direct tissue contraction which prevented assessment of the amplitude of neurogenic responses.

In three tissues the depressant effect of dopamine was retested following addition to the bath of haloperidol in a final concentration of 0.1 μM (two experiments) or 0.19 μM (three experiments). Under these conditions the effect of dopamine was enhanced (Table 1). In five further experiments, phentolamine (0.27 μM) greatly reduced the effect of dopamine (Table 1) while having no effect on responses to nerve stimulation itself.

Haloperidol had no effect on the amplitudes of responses to either nerve stimulation or exogenous noradrenaline (10 μM applied for 30 s every 150 s) (Table 2).

Discussion

We have demonstrated that depression of vasoconstrictor responses to adrenergic nerve stimulation of the rabbit ear artery by exogenous dopamine accompanies the depression of tritiated noradrenaline overflow reported by McCulloch *et al.* (1973). As the effect of dopamine was antagonized by the dopamine-receptor antagonist haloperidol at a concentration that lacked α-adrenoceptor blocking activity, but was not antagonized by phentolamine at a concentration sufficient to exert some post-synaptic α-adrenoceptor antagonism, it can be attributed to an action at specific dopamine receptors.

Inhibition of vasoconstrictor responses could occur if dopamine activated inhibitory receptors on the vascular muscle cells. However, this does not seem to be a possible explanation of the present results, as we could not demonstrate any direct relaxant effect of dopamine in the ear artery. It is likely, therefore, that the depressant effect of dopamine on neurogenic vasoconstriction is due to a presynaptic action on axonal conduction or transmitter release.

There is some evidence to suggest that the rabbit ear artery may possess a dilator nervous supply in addition to the adrenergic vasoconstrictor innervation (Holton & Perry, 1951; Kalsner, 1974). The transmitter involved in the mediation of this dilator response is unknown, but from published data seems unlikely to be acetylcholine, histamine, an adenyl nucleotide or a prostaglandin. Our demonstration of the absence of dilator receptors for dopamine on the muscle cells of the artery make dopamine also an unlikely candidate for a role in this response.

We obtained no evidence to support the presence of

Table 2 Effect of haloperidol on excitatory responses of rabbit ear artery and guinea-pig vas deferens to adrenergic nerve stimulation (2 Hz) and to applied noradrenaline (artery, 0.1 nmol intraluminally; vas deferens 10 μM).

Tissue	*Haloperidol* (μM)	*Nerve stimulation* *Control*	*Haloperidol*	*Noradrenaline* *Control*	*Haloperidol*
Rabbit ear artery	0.1	99 ± 23 mmHg	88 ± 19 mmHg	67, 73 mmHg	67, 87 mmHg
		(*n* = 9)		(*n* = 2)	
Guinea-pig vas deferens	0.1	168, 441 mg	169, 397 mg	–	–
		(*n* = 2)			
	0.19	273 ± 64 mg	290 ± 70 mg	70, 76 mg	64, 73 mg
		(*n* = 6)		(*n* = 2)	

presynaptic dopamine-receptors in the guinea-pig vas deferens. In this tissue the depressant action of dopamine on nerve-mediated responses appeared to be solely through activation of α-adrenoceptors, as it was antagonized by phentolamine but unaffected by haloperidol. This difference could be due to species variation. Alternatively it could be related to the differing dimensions of the neuromuscular junctions in the two tissues. In the vas deferens, axons which lie within 200 Å of a muscle cell are common (Merillees, Burnstock & Holman, 1963), while in arteries a minimum axon-muscle separation of at least 800–1000 Å is usual (Bell, 1969; Burnstock, 1970). If the presynaptic amine receptors were to have any physiological function then their distribution might be expected to vary between junctions with different dimensions and therefore different extracellular transmitter kinetics. One piece of evidence against such a postulate is a recent report that inhibition of nerve-mediated contractions of rat vas deferens is antagonized by dopamine-receptor antagonists (Tayo, 1977). As the neuromuscular organization of the rat vas deferens more closely resembles that of the guinea-pig vas deferens than that of arteries (Richardson, 1962; Taxi, 1965), this result would tend to favour species variation as the basis for the difference between tissues seen in our experiments.

Although haloperidol antagonized the depressant effect of dopamine on neurogenic responses in the ear artery, it did not increase the amplitude of the nerve-mediated responses themselves in either this tissue or the guinea-pig vas deferens. This result does not support the theory that presynaptic depression by endogenous dopamine modulates adrenergic transmission at physiological firing frequencies.

This work was supported by the National Heart Foundation of Australia.

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(Received February 28, 1977.
Revised May 3, 1977.)

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ELECTROCORTICAL CHANGES INDUCED BY THE PERFUSION OF NORADRENALINE, ACETYLCHOLINE AND THEIR ANTAGONISTS DIRECTLY INTO THE DORSAL RAPHÉ NUCLEUS OF THE CAT

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1 The electrocortical changes induced by the perfusion of drugs directly into the dorsal raphé nucleus of the cat *encéphale isolé* preparation have been studied.

2 (–)-Noradrenaline (NA), (–)-adrenaline, or (–)-isoprenaline (Isop) produced intermittent or sustained electrocortical desynchronization during the perfusion period.

3 These changes were markedly attenuated or completely abolished by the prior perfusion of (±)-sotalol or (–)-propranolol, but not by equimolecular concentrations of (+)-propranolol.

4 The effects of NA or Isop were also blocked by phentolamine, whereas phenoxybenzamine either potentiated the responses to NA and Isop or mimicked the effects of these catecholamines.

5 The effect of dopamine was similar to that induced by NA, but could not be elicited at all of the perfusion sites where NA was effective. It could be blocked by (±)-sotalol or (–)-propranolol and also by the prior perfusion of fusaric acid.

6 Acetylcholine (ACh) increased, or initiated, electrocortical synchronization. These effects could be antagonized by sensory stimulation, the prior perfusion of atropine, or the simultaneous perfusion of NA at the same site.

7 Lignocaine, induced prolonged electrocortical desynchronization, behavioural alerting and an increased responsiveness to sensory stimulation.

8 The results have been discussed in relation to the possible involvement of inhibitory β-adrenoceptors and facilitatory cholinoceptors (muscarinic) in the functioning of the dorsal raphé nucleus.

Introduction

Of the rostrally projecting midline raphé nuclei, nucleus raphé dorsalis (NRD) possesses a high density of 5-hydroxytryptamine-containing cells (Dahlstrom & Fuxe, 1965) and contributes a significant proportion of its fibres to the forebrain and, in particular, to neocortical structures (Lorens & Guldberg, 1974). It is thus well suited anatomically to fulfil a major role in the modulation of electrocortical activity. Indeed, although lesions confined to this structure do not produce the severity of behavioural deficit incurred on destruction of other raphé nuclei (Srebro & Lorens, 1975), the NRD would appear to play an important part in electrocortical synchronization and sleep mechanisms (Morgane & Stern, 1972; Jouvet, 1973).

In view of these observations it was thought worthwhile to study the influence on the arousal level and patterns of electrocortical activity of substances applied directly to neurones within the DRN through stereotactically implanted perfusion cannulae. A number of putative transmitter substances have been implicated in the functioning of the raphé system (Couch, 1970) and fluorescence and histochemical studies have indicated that the nuclei receive both noradrenergic and cholinergic afferent inputs (Lewis & Shute, 1967; Chu & Bloom, 1974; Saavedra, Grobecker & Zivin, 1976). The present paper therefore deals with the effects of noradrenaline (NA) and acetylcholine (ACh) and their respective antagonists, in an attempt to determine their role within the DRN and the character of the receptors involved.

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Methods

A total of 29 cat *encéphale isolé* preparations were used. The operative procedure, carried out under halothane/O_2 anaesthesia, the type and position of the cortical recording electrodes, as well as the implantation technique have been described previously (Bradley & Elkes, 1957). A concentric, two-tube perfusion cannula (Key, 1975), angled at 45°, was inserted stereotactically using co-ordinates from the cat brain atlas of Snider & Niemer (1961), so that the tip of the outer cannula lay just below the ventricular surface in the dorsal aspect of the nucleus raphé dorsalis. The area of perfusion at the tip of the cannula was 0.53 mm^2. Drugs were dissolved in artificial cerebro-spinal fluid (CSF) and perfused at a rate of 120 µl/min with a 20-channel Watson-Marlow H.R. Flow Inducer. The artificial CSF was perfused continuously throughout the experiment and the drug solutions switched in for 5 min periods.

Systemic blood pressure was monitored by means of a mercury manometer connected to the femoral artery. Wound edges and pressure points were infiltrated with 1% w/v lignocaine hydrochloride solution upon completion of all operative procedures and subsequently at intervals throughout the experiment. The animal was then released from the stereotactic apparatus, the anaesthetic discontinued and a period of 1 h allowed for recovery from the effects of the anaesthetic. Control recordings of electrocortical activity were taken over a further hour before the perfusion of artificial CSF was started.

The electrocorticogram (ECoG) was monitored throughout the experiment and for descriptive purposes reference has been made to four basic patterns representing the alert, relaxed, drowsy and sleeping behavioural states (Figure 1b) according to the schema described by Bradley & Elkes (1957). Rapid eye movement (paradoxical) sleep was not observed in these preparations. A more quantitative assessment of the changes produced in the ECoG was carried out by integrating the electrocortical waveform recorded from the association cortex of the left or right middle supra-sylvian gyrus. The output of the integrator was in the form of pulses representing fixed increments of electrical energy. These pulses were recorded on the ECoG coincident with the appopriate electrical activity (Figure 1b-int). The data were further analysed by counting the number of pulses for each consecutive 20 s period and expressing these cumulative integrals graphically to reflect the changing levels of desynchronization of the ECoG (Figure 1a). Upon completion of the experiment the brain was removed and fixed in 10% formol saline solution for later histological investigation.

All experiments were carried out in the open laboratory where levels of ambient, and especially auditory, stimulation varied. Since this factor appeared to be important in the intrepretation of the results, an assessment of this variation was made at the time of the experiment. Under 'quiet' conditions sound levels rarely exceeded 20 dB (measured relative to 0.0002 dynes cm^2 at the position of the animal). Moderate levels, usually of continuous noise varied up to 50 dB, but occasionally higher levels of an intermittent nature were registered above this level.

The drugs tested were atropine sulphate (BDH), acetylcholine chloride (BDH), phentolamine mesylate (Ciba), fusaric acid (Sigma), and the hydrochlorides of (–)-adrenaline (Sigma), dopamine (Koch-Light), (–)-isoprenaline (Sigma), (–)-noradrenaline (Sigma), phenoxybenzamine (SKF), (–)- and (+)-propranolol (ICI), (±)-sotalol (Mead-Johnson) and lignocaine (Antigen).

Results

Control recordings of electrocortical activity showed that no observable changes in the patterns occurred when the temperature of the perfusate was maintained at 38° ± 1.5°C and the pH between 5.7 to 7.2. The tube from the output cannula was open-ended and the flow of perfusate maintained by the positive pressure of the pump. Because the tip of the inner input tube was 0.5 mm shorter than the outer tube, the resistance to the flow of perfusate from input to output systems was never greater than 1 mmHg. Thus, brain deformation at the tip of the cannula was insigificant and dye studies carried out at the end of the experiments indicated that although in each case the cannulae were in the midline and only 0.5–0.75 mm below the ventricular surface, no leakage of perfusate occurred back along the outside of the cannula into the aqueduct or fourth ventricle. In the experiments which are described all the above criteria were fulfilled and histological examination showed that tissue damage was limited to that incurred on insertion of the cannula.

Noradrenaline

Noradrenaline (NA) in the concentration range 10^{-6}–10^{-3}M was perfused in 29 cats. In the drowsy or sleeping animal, where the ECoG showed varying degrees of synchronization, perfusion of NA above 10^{-1}M always induced some measure of desynchronization. These effects are illustrated in Figure 1, where an increase in electrocortical desynchronization is reflected by a decrease in the integral counts. The effects of NA were dose-related. Concentrations of 10^{-5}M induced slight increases in the frequency of electrocortical activity, whereas concentrations of 5×10^{-5}M or above produced short, 1–10 s, periods of desynchronization (phasic arousal responses) after 40–90 s of perfusion. This pattern then either persisted for the duration of the perfusion or was replaced by prolonged desynchronization associated

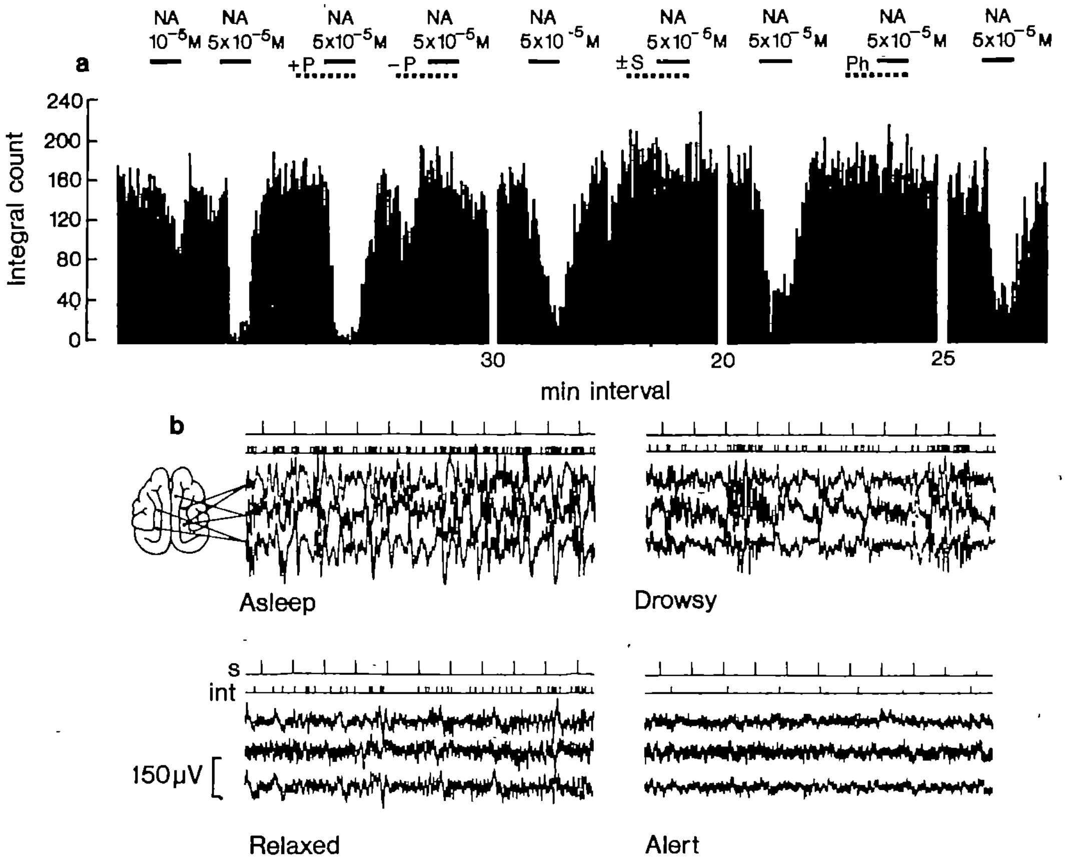

Figure 1 The effect of noradrenaline (NA) on the pattern of electrocortical activity when perfused into the dorsal raphé nucleus of the cat. (a) Histogram depicting the integral counts over successive 20 s periods. Solid bars show 5 min perfusion periods of NA. Perfusion of NA antagonists indicated by the interrupted bars. Desynchronization of the electrocorticogram, recorded from the right middle suprasylvian gyrus is reflected by a decrease in the integral counts. Note the dose-related changes induced by NA with the subsequent blockade of the response by (−)-propranolol (−P, 5×10^{-5} M), (±)-sotalol (S, 5×10^{-5} M) and phentolamine (Ph, 5×10^{-4} M) but not by (+)-propranolol (+P, 5×10^{-5} M). In this experiment (−)-propranolol induced a transient desynchronization of the ECoG at the beginning of the perfusion period. (b) Examples of the four basic patterns of electrocortical activity referred to in the text with the coresponding integral pulses (int). Integration was carried out on the electrical record taken from the right middle suprasylvian gyrus (top trace in each record).

with behavioural arousal (tonic arousal response). However, the appearance of tonic responses could be blocked or at least delayed, by a reduction in the level of ambient sensory stimulation. Recovery from the effects of NA were extremely variable. The majority of animals returned to the sleeping state within 1–10 min, while others, once tonic arousal had occurred, remained alert or in the relaxed behavioural state, for up to 40 minutes.

Perfusion of adrenaline (3 expts) or isoprenaline (Figure 2—Isop, 10 experiments) produced effects comparable to those of NA, but the minimal effective concentrations were slightly higher (5×10^{-5} M). Tachyphylaxis occurred to all three drugs, especially with high concentrations (10^{-3} M) and when the drugs were applied at intervals shorter than 5 minutes. No consistent blood pressure changes were noted on perfusion of adrenaline, NA or Isop.

Adrenoceptor antagonists

The β-adrenoceptor antagonists (−)-propranolol and (±)-sotalol blocked or significantly attenuated the phasic and tonic electrocortical responses induced by both NA and Isop. Both drugs showed similar antagonistic activity and were effective in concentrations as low as 10^{-5} M, providing they were applied during, and at least 5 min before the perfusion

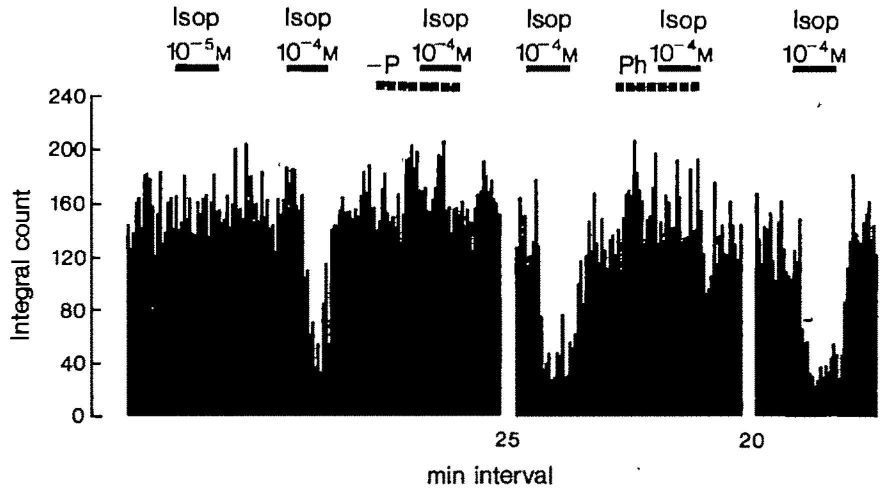

Figure 2 Histogram showing the effect of isoprenaline (Isop) on the pattern of electrical activity when perfused into the dorsal raphé nucleus of the cat. Integral counts taken over successive 20 s periods using electrocortical activity of the right middle suprasylvian gyrus taken from the same experiment as in Figure 1. Solid bars indicate 5 min perfusion periods of isoprenaline. Perfusion of antagonists shown by interrupted bars (15 minutes). Note response of Isop blocked by (–)-propranolol (–P, 5×10^{-4} M) and phentolamine (Ph, 5×10^{-4}M).

of NA or Isop (Figures 1 and 2). Basic differences in the responses of the two antagonists were apparent, since (–)-propranolol appeared to possess some noradrenergic agonist activity. In the sleeping animal concentrations of at least 5×10^{-5} M occasionally produced an overall increase in the frequency of electrocortical activity or induced short periods of desynchronization. These effects were transient and usually only apparent in the first 1–3 min of the perfusion as shown by a decrease in the cumulative integral count (Figure 1a). It is unlikely that these changes are related to local anaesthetic activity, since an equimolar concentration of the (+)-isomer of propranolol had no effect (Figure 1a). Indeed, by comparison with the responses induced by the local anaesthetic lignocaine (Figure 6), the concentrations of (–)- or (+)-propranolol that appeared to have local anaesthetic activity and produce long-lasting electrocortical desynchronization, had to be at least 10^{-3} M. This effect then persisted for 10–30 min after the end of the perfusion period.

Phenoxybenzamine (10^{-6}–10^{-4} M) failed to antagonize the response induced by NA. At a concentration of 10^{-5} M this drug induced short 1–5 s bursts of electrocortical desynchronization and even at 5×10^{-6} M potentiated rather than blocked the effect of subsequent applications of NA or Isop. With concentrations in excess of 10^{-5} M phenoxybenzamine produced tonic electrocortical and behavioural arousal responses. In contrast, phentolamine (10^{-5}–10^{-3} M) had no effect on the ECoG but when used in concentrations of 10^{-4} M or above, consistently attenuated or completely blocked the responses induced by NA and Isop (Figures 1 and 2). However, when the antagonistic action of (–)-propranolol was compared with that of phentolamine in the same preparation (Figure 1), it was always found to be at least 10 times more potent as a blocking agent.

Dopamine

Dopamine was perfused in 12 experiments but produced changes in the ECoG in only 8. The electrocortical effects were similar to those of NA (Figure 3), but noticeably, much higher perfusate concentrations (10^{-4}–5×10^{-3}M) were required and consecutive applications, even at widely spaced time intervals, did not yield the same degree of electrocortical desynchronization. Although these observations do not exclude the participation of post-synaptic dopamine receptors, the longer latency and inconsistent nature of the response appeared to favour a non-specific or indirect effect, possibly mediated through NA. This latter hypothesis was investigated using the β-adrenoceptor antagonists, (–)-propranolol and (±)-sotalol, both of which proved effective in 10^{-5}M concentrations in abolishing the electrocortical changes induced by application of dopamine. In addition, prior perfusion for 15 min of the dopamine β-hydroxylase inhibitor, fusaric acid, at a concentration of 10^{-5} M, also blocked the effect of dopamine but not that of NA (Figure 3).

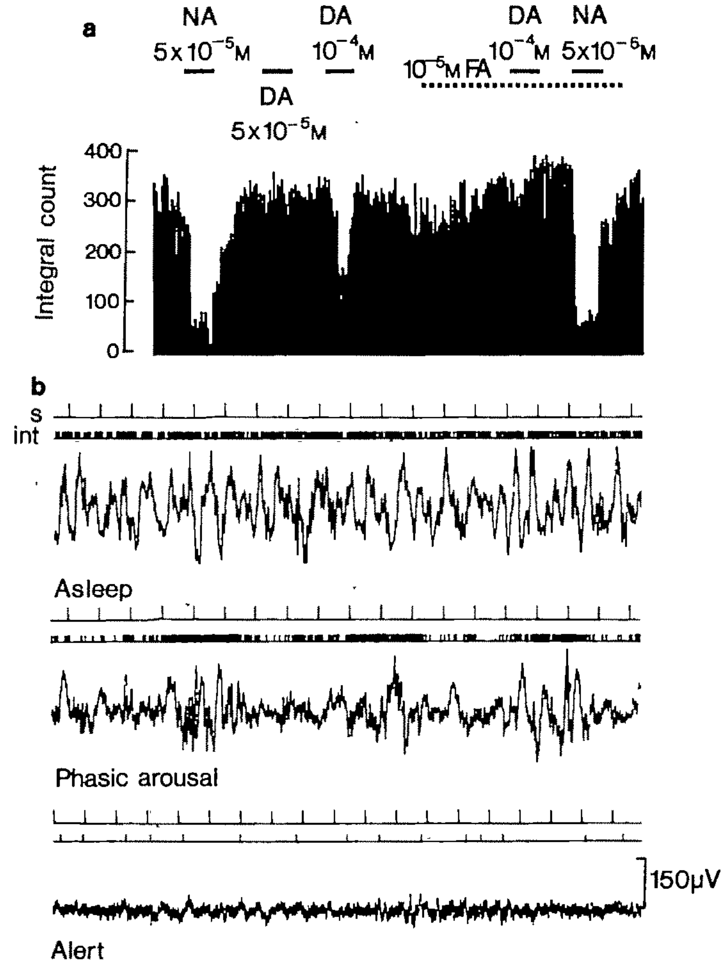

Figure 3 Comparison between the electrocortical changes induced by noradrenaline (NA) and dopamine (DA) when perfused into the same site within the dorsal raphé nucleus of the cat. (a) Histogram showing integral counts over successive 20 s periods. Electrical activity recorded from the right middle suprasylvian gyrus. Solid bars show 5 min perfusion periods of either NA or dopamine. Perfusion of the dopamine β-hydroxylase inhibitor, fusaric acid (FA 10^{-5} M) indicated by the interrupted bars (total time 33 minutes). Note the blockade of the desynchronization induced by dopamine but not that produced by NA. (b) Sample electrocortical records taken from the right middle suprasylvian gyrus with concomitant integral pulses (int). Record of electrocortical activity when the animal was asleep taken immediately before the initial perfusion of dopamine at 10^{-4} M. Phasic arousal responses were obtained during the perfusion of dopamine (10^{-4} M) and the fully desynchronized record (alert) shows the effect of initial perfusion of NA (5×10^{-5} M).

Acetylcholine

The effect of acetylcholine (ACh) was studied in 15 preparations and found to be opposite to that of NA. To produce a change in electrocortical activity it was found necessary to perfuse ACh in high concentrations (10^{-3}–10^{-1} M). If physostigmine (10^{-5} M) was added to the perfusage (3 experiments), the effective concentrations of ACh were considerably lower (10^{-5}–10^{-4} M). However, in view of the observations of Bradley, Dhawan & Wolstencroft (1966) that some apparently non-cholinoceptive neurones within the CNS respond to iontophoretically applied physostigmine, it was decided to use ACh alone and thus eliminate any misinterpretations which might rise due to the addition of an extra drug. Although ACh was capable of initiating or increasing electrocortical synchronization in the alert animal, the degree of electrical change was determined by the level of ambient sensory stimulation at the time of the perfusion. With high environmental noise levels, especially if the auditory stimuli were of an

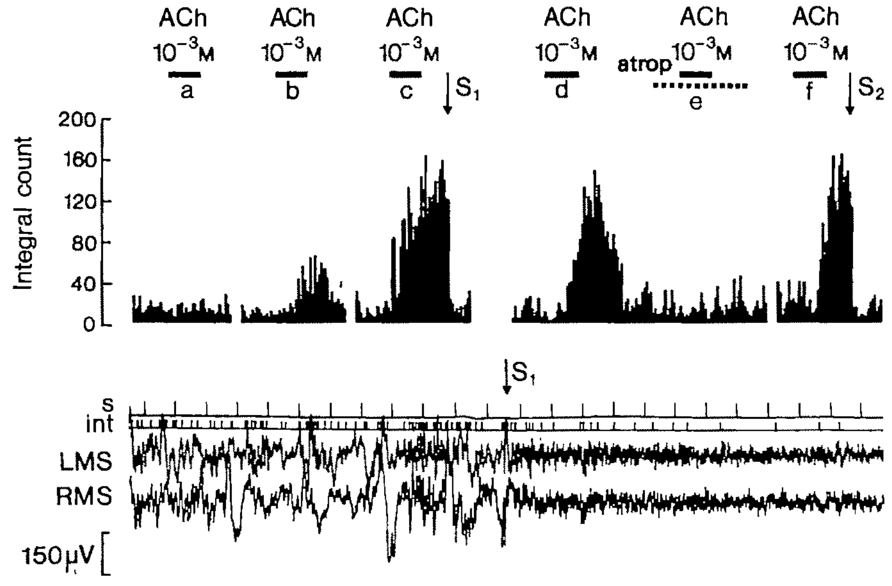

Figure 4 The effect of acetylcholine (ACh) on the patterns of electrocortical activity when perfused into the dorsal raphé nucleus of the cat. Histogram represents integral counts over successive 20 s periods of electrical activity recorded from the left middle suprasylvian gyrus. Solid bars indicate 5 min perfusion periods of ACh. Interrupted bars represent 15 min perfusion period of atropine (Atrop, 10^{-4} M). (a) Lack of effect of ACh when perfused at a time of high ambient noise levels in the immediate environment. (b and c) Electrocortical synchronization induced by the perfusion of ACh with the animal in an environment providing only low to moderate (15–40 dB) levels of ambient noise. (c) Blockade of the electrocortical synchronizing effect of ACh (d) by atropine (Atrop, 10^{-4} M) with subsequent recovery (f). Note that electrocortical desynchronization can easily be evoked by an auditory stimulus (S_1 and S_2) after ACh had produced synchronization.

intermittent nature, ACh appeared to be without effect (Figure 4a). On the other hand, low to moderate levels of noise (15–40 dB), which were usually sufficient to maintain wakefulness before drug application, proved insufficient after the perfusion of ACh. The changes in the ECoG varied from the introduction of rhythmic (8–12 Hz) activity characteristic of the relaxed behavioural state (Figure 4b), to the initiation of high amplitude, slow waves (150–300 µV, 2–4 Hz) associated with behavioural sleep (Figure 4c).

The effects of ACh could be blocked either by the simultaneous perfusion of NA (10^{-4} M—4 experiments), or the prior perfusion of atropine (5 experiments) at concentrations of 5×10^{-5} M or 10^{-4}M (Figure 4e). The dose-response characteristics for atropine (Figure 5) revealed that at these levels the drug did not exert any significant local anaesthetic activity. In the sleeping animal a concentration of 5×10^{-5} M had no effect on the ECoG (Figure 5). At 10^{-4}M there was a decrease in the cumulative integral count, induced as a result of the introduction of phasic, 5–10 s bursts of desynchronization (Figure 5). This change was not accompanied by any overt behavioural response and terminated quickly (1–3 min) after the end of the perfusion. In contrast, application of concentrations in excess of 5×10^{-4} M (Figure 5) invariably induced prolonged, tonic electrocortical desynchronization and behavioural arousal, occasionally of rather sudden onset (Figure 5–10^{-3} M).

Lignocaine

Since a number of the drugs used in the present study have been reported to possess local anaesthetic activity, especially in high concentrations, it was thought worthwhile to study the effect of the local anaesthetic, lignocaine. Such observations would also provide an indication of the electrocortical and behavioural changes accompanying reversible blockade of the NRD in the *encéphale isolé* preparation. Lignocaine produced tonic electrocortical desynchronization and behavioural arousal within the 5 min perfusion period when applied in concentrations of 10^{-5} M, or above. The effects were usually of sudden onset, long-lasting and with the higher concentrations, characterized by a very short latency (Figure 6–10^{-4} M). It was also noticeable that the animals showed increased responsiveness to sensory stimuli and would respond with head, eye and ear movements to the slightest sound, movement or tactile stimulus. Reduction in the level of ambient

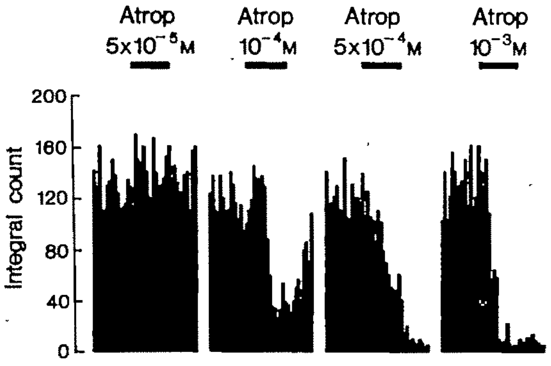

Figure 5 Dose-related changes in the degree of electrocortical desynchronization induced by atropine (Atrop) when perfused into the dorsal raphé nucleus of the cat. Histogram shows integral counts representing successive 20 s periods of electrical activity recorded from the left middle suprasylvian gyrus. Same experiment as in Figure 4. Solid bars represent 5 min perfusion periods of atropine with 20 min recovery periods between each dose. Note response to 5×10^{-4} M atropine shows a two stage effect, while at 10^{-3} M the response has a sudden onset and relatively short latency.

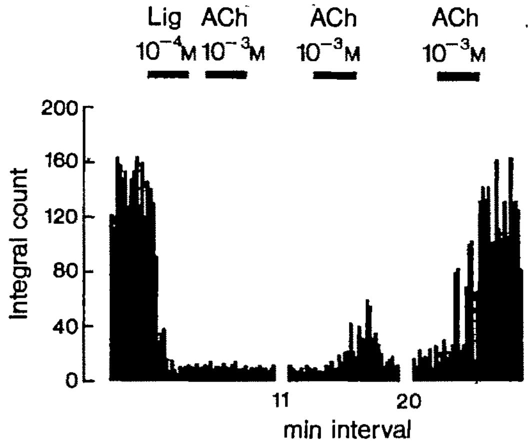

Figure 6 Histogram showing the desynchronizing effect of lignocaine (Lig) when perfused for 5 min into the dorsal raphé nucleus of the cat. Same experiment and perfusion site as in Figures 4 and 5. Note the sudden onset of the response and the blockade of the electrocortical synchronizing effect of acetylcholine (ACh).

sensory stimulation, although usually failing to modify the response once present, invariably delayed its onset.

Lignocaine blocked the synchronizing effects of ACh and recovery, as can be seen from Figure 6, was delayed. Partial ACh responses were apparent after 11 min but full recovery, following a 10^{-4} M concentration of lignocaine, was only seen after a further 20 min (Figure 6).

Discussion

The present study shows that it is possible to influence the arousal level and the pattern of electrocortical activity by application of drugs directly to the dorsal raphé nucleus. NA invariably produced some degree of electrocortical desynchrony when perfused in concentrations of 10^{-5}–10^{-3} M. When considered in terms of the total amount of NA applied during the 5 min perfusion period, these concentrations represent relatively substantial doses. However, similar perfusion studied within the mesencephalic reticular formation have shown that only a fraction of the applied NA, in the order of 0.39–119.2 ng respectively, will actually penetrate into the brain tissue (Key, 1975). On this evidence it would appear that the diffusibility of NA is either very low or that the use of a continuous perfusion process reduces the amount of NA which physically comes into contact with the brain. In either case the amount of NA within the vicinity of individual neurones, especially those located towards the limit of the diffusional area, must be relatively small.

Biochemical, stimulation and lesion studies have indicated that the raphé nuclei play a part in sleep processes and that it is activation of this system which initiates or increases the synchronization of electrocortical activity (Kowstowski, Giacalonne, Garattini & Valzelli, 1969; Morgane & Stern, 1972; Jouvet, 1973). The desynchronization induced by the perfusion of NA would suggest, by analogy, that the predominant effect of this drug within the DRN is one of inhibition. It is also likely on the basis of the results obtained with Isop and adrenaline, that this inhibitory effect is related to β-adrenoceptor activation. The failure of phenoxybenzamine to block the desynchronization induced by NA and Isop would appear to substantiate this view. Even in relatively low concentrations phenoxybenzamine produced changes in the ECoG qualitatively similar to those of NA. Moreover, pretreatment with low concentrations of phenoxybenzamine potentiated rather than blocked the effects of NA or Isop. It has been reported that phenoxybenzamine can facilitate the release (Potter, Chubb, Put & Schaepdryver, 1971), or inhibit the uptake of catecholamines into adrenergic nerve terminals (Iversen, Salt & Wilson, 1972). Both effects, since they are related to pre-synaptic events, would be expected to facilitate the effect of exogenously applied NA and Isop, especially at β-adrenoceptor sites where the antagonistic effects of phenoxybenzamine should be negligible.

Surprisingly, both the β-antagonist, (–)-propranolol and the α-antagonist, phentolamine, attenuated or abolished the responses induced by NA and Isop. Local anaesthetic effects may be excluded at the dose levels employed since (+)-propranolol, which has greatly reduced β-adrenoceptor blocking activity but is equipotent with the (−)-isomer in terms of local anaesthetic activity (Barrett & Cullum, 1968), did not affect the ability of NA or Isop to produce desynchrony. Moreover, the β-antagonist (±)-sotalol, which is purported to have little or no local anaesthetic activity (Lish, Weikel & Dungan, 1965), was just as effective as (−)-propranolol as a blocking agent. The similarity in effect between phentolamine and the β-antagonists would imply either that it is difficult to categorize the catecholamine receptors in the raphé on the basis of criteria used for the peripheral nervous system, or more likely that the effect of phentolamine, although exerted on β-receptors, was non-specific. Indeed, by comparison with propranolol and sotalol, it was noticeable that higher concentrations of phentolamine were always required. Recently it has been pointed out that catecholamine receptor designation based solely on phentolamine inhibition is to be approached with caution. This drug, especially in high concentration is capable of displacing the specific β-noradrenergic antagonist (−)-alprenolol, from post-synaptic binding sites (Alexander, Davis & Lefkowitz, 1975). In addition, phentolamine is known to produce non-specific inhibitory effects on certain β-adrenergic physiological responses (Moran & Perkins, 1961) and on β-adrenoceptor-coupled adenylate cyclase activity (Vatner & Lefkowitz, 1974).

Although there are no reports of dopamine-containing terminals within the DRN, or any direct evidence of afferent fibre projections to the DRN from dopamine-containing neurones, it was still possible by the perfusion of dopamine to produce electrocortical desynchronization. This effect was not observed in all experiments and only after doses substantially higher than those of NA. It could be blocked by the application of (−)-propranolol or (±)-sotalol, and the prior perfusion of the dopamine β-hydroxylase inhibitor, fusaric acid (Nagatsu, Tekeshi & Hiroshi, 1973), abolished the response in all cases. On this evidence it may be concluded that the dopamine effect was mediated through NA post-synaptic receptor activation but involved initially the pre-synaptic enzymatic conversion of dopamine to NA.

Provided the animal was kept in an environment yielding only a low to moderate level of ambient sensory stimulation, the perfusion of ACh initiated, or increased the degree of electrocortical synchronization. In this respect the response induced by ACh is comparable to that evoked by low frequency electrical stimulation of the raphé system (Kowstowski *et al.*, 1969). However, the effect could be blocked or attenuated by the simultaneous perfusion of NA or by the prior perfusion of atropine. These results would suggest that the cells of the DRN receive a noradrenergic inhibitory input, physiologically antagonistic to an afferent excitatory cholinergic system. On the basis of the blocking action of atropine the post-synaptic cholinoceptors may be classified as of the muscarinic type. Cholinergic involvement in the functioning of the raphé nuclei has been indicated previously by a number of histological and histochemical studies (Lewis & Shute, 1967; Palkovits & Jacobwitz, 1974; Cheney, LeFevre & Racagni, 1975). Recently it has also been shown that [^{3}H]-quinillidinyl-benzilate (QNB), a potent cholinoceptor antagonist, bind specificially to muscarinic post-synaptic receptors (Yamamura, Kuhar & Snyder, 1974). Within the midbrain, binding has been observed in the raphé area and in particular within the dorsal midbrain raphé (Kuhar & Yamamura, 1975).

The behavioural and electrical changes produced by the perfusion of NA and ACh into the DRN were clearly related to environmental conditions. On the evidence presented, the effect of NA was predominantly one of inhibition. Even so, in the sleeping animal blockade of dorsal raphé activity was usually insufficient to bring about tonic behavioural and electrocortical arousal unless some degree of ambient sensory stimulation was present. Since this level of stimulation was ineffective before drug application, it would appear that the alerting was induced by the same sensory input to which the animal, as a result of raphé inhibition, had become more susceptible. In this context it is worth noting that the raphé nuclei have neural connections, or exert inhibitory influences, not only on the hypothalamus, neocortex, limbic system and the reticular formation, but also on structures concerned with the specific sensory systems (Fuxe, 1965; Nakamura, 1975; Bobillier, Sequin, Petitjean, Salvert, Touret & Jouvet, 1976). Moveover, the raphé nuclei have been implicated in behavioural functions related to changes in sensory responsiveness or the integration of sensory information (Sheard & Aghajanian, 1969; Geyer, Puerto, Menkes, Segal & Mandell, 1976). The raphé nuclei therefore, instead of forming part of an active sleep mechanism, may provide a system which controls the flow and integration of sensory information. Sleep, induced as a result of raphé activation, could thus be an indirect effect initiated by the modulation of sensory inflow and the establishment of conditions conducive to the production of sleep.

We would like to thank Mr T. Beckett for his technical assistance, Mr R.W. Blunn of the M.R.C. Neuropharmacology Research Unit, Medical School, Birmingham, for the design and construction of the EEG Integrator and Professor P.B. Bradley for his interest in the work. L.K. was the recipient of a W.H.O. Fellowship.

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(Received March 2, 1977.
Revised April 6, 1977)

Br. J. Pharmac. (1977), **61**, 307–313

EFFECTS OF MIANSERIN, A NEW ANTIDEPRESSANT, ON THE *in vitro* AND *in vivo* UPTAKE OF MONOAMINES

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1 The effects of mianserin and of selected tricyclic antidepressants were compared in a number of monoamine uptake models.

2 The ability of mianserin to block the noradrenergic neurone membrane amine pump of rabbit brain stem slices was comparable to that of imipramine and amitriptyline and less than that of desipramine and nortriptyline. Both mianserin and desipramine were competitive inhibitors of noradrenaline uptake *in vitro*. The effect of mianserin on noradrenaline uptake *in vivo* was studied both peripherally and centrally. The ability of 6-hydroxydopamine to lower rat heart noradrenaline levels was found to be very sensitive to inhibition by tricyclic antidepressants. Mianserin was active in this model. However, its ability to block the 6-hydroxydopamine-induced fall in rat heart noradrenaline concentration was appreciably less than that of the tricyclics studied.

3 Mianserin, like tricyclic antidepressants, was essentially devoid of effect on dopamine uptake both *in vitro* and *in vivo*.

4 The ability of mianserin to inhibit $[^3H]$-5-hydroxytryptamine uptake by rat hypothalamic synaptosomes was appreciably less than that of the tricyclic antidepressants studied. Mianserin was essentially devoid of effect on rat brain 5-hydroxytryptamine uptake *in vivo*.

5 It is concluded that in certain situations large doses of mianserin may block noradrenaline uptake *in vivo*. However, in no way does mianserin rival tricyclic antidepressants in blocking monoamine uptake *in vivo*. The clinical efficacy of mianserin cannot be attributed to inhibition of monoamine uptake.

Introduction

The primary selection of potential antidepressants is often based on the ability of the agent to antagonize reserpine-induced hypothermia and/or its ability to block ptosis induced by drugs such as reserpine and tetrabenazine (Spencer, 1976). Activity in such models is usually attributed to a drug-induced blockade of the so-called membrane amine pump of central monoaminergic neurones. Furthermore, the clinical efficacy of compounds such as the tricyclic antidepressants is widely attributed to their ability to block central monoaminergic uptake mechanisms (Carlsson, 1976). Mianserin (Org GB94, 1, 2, 3, 4, 10, 14b-hexahydro-2-methyl-dibenzo [*c,f*]pyrazino-[1,2]-azepine monohydrochloride, Figure 1) is a clinically effective antidepressant (Itil, Polvan & Hsu, 1972; Coppen, Gupta, Montgomery, Ghose, Bailey, Burns & de Ridder, 1976). However, mianserin does not antagonize reserpine-induced hypothermia (van Riezen, 1972). Moreover, neurochemical experiments have revealed that mianserin, in contrast to the tricyclic antidepressants, increases the turnover of noradrenaline in the rat brain (Kafoe, de Ridder & Leonard, 1976). The objective of this study was to determine what effects, if any, mianserin exerts on *in vitro* and *in vivo* monoamine uptake mechanisms. Tricyclic antidepressants were included for comparative purposes. A preliminary account of some of the findings has been published in abstract form (Goodlet & Sugrue, 1974).

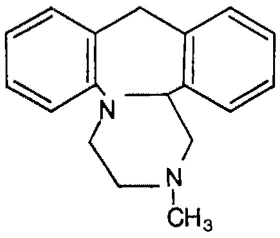

Figure 1 Structure of mianserin.

Methods

Animals used were either adult New Zealand white rabbits weighing 3.5–4.0 kg or male Wistar rats (CE/CFHB strain) weighing 200–250 grams.

In vitro *studies*

In vitro blockade of the membrane amine pump of central noradrenergic neurones was investigated by determining drug effects on the uptake of (–)-metaraminol ((–)-MA) by rabbit brain stem slices. The rabbit was killed and its brain quickly removed and immersed in ice-cold 0.9% w/v NaCl solution (saline). The brain was placed ventral side uppermost on a dissecting plate and a transverse cut was made at the level of the optic chiasma. Sagittal cuts were made on both sides at the medulla oblongata and pons to the level of the optic chiasma and a wedge of tissue comprising the hypothalamus, pons and medulla oblongata, subsequently referred to as the brain stem, was dissected free from the remainder of the brain. Ventral slices of about 0.3 mm thickness were obtained by means of a Harvard tissue slicer. Slices of 80–110 mg were placed in 5 ml of ice-cold Krebs-Ringer phosphate buffer (pH 7.4) (for composition see Sugrue & Shore, 1969) in 25 ml beakers which were then placed in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% O_2 and 5% Co_2. (–)-MA, in a volume of 0.1 ml in buffer, was added after a 15 min pre-incubation period and incubation was continued for 30 min, uptake being linear with respect to time. Following incubation, slices were removed, rinsed in ice-cold saline and superficial saline was removed by means of tissue paper. After weighing, tissues were homogenized in 0.4 N perchloric acid. The fluorometric procedure of Shore & Alpers (1964) was used to analyse the slice content of (–)-MA. Amine uptake was calculated as net uptake, concentration per ml of slice water minus medium concentration, on the basis of a tissue water content of 85%, as described elsewhere (Giachetti & Shore, 1966). Drugs under study were added in a volume of 0.1 ml at the start of the pre-incubation period.

In experiments with rabbit hypothalamic and striatal minces, brain areas were dissected out as described by Glowinski & Iversen (1966). The dissected brain areas were placed in ice-cold saline and then minced as finely as possible with iris scissors (Dorris & Shore, 1971). The minces were washed in ice-cold saline before incubation. The weight of the minces averaged 25–30 mg. The remainder of the procedure was as described above.

In vitro uptake of 5-hydroxytryptamine (5-HT) was studied with a crude synaptosomal fraction from rat hypothalamus which was dissected out as described by Glowinski & Iversen (1966). The tissue was weighed and homogenized in 19 volumes of 0.32 M sucrose with five upward and downward strokes of a motor driven teflon pestle, clearance 0.1 mm. The resultant homogenate was centrifuged at 1000 *g* for 10 minutes. The supernatant liquid was decanted and stirred to give a uniform suspension. A 0.1 ml aliquot of suspension (amount of tissue present was equivalent to 5 mg of original tissue) was added to 25 ml beakers containing 2 ml of Krebs-bicarbonate buffer (for constituents, see Coyle & Snyder, 1969) containing ascorbic acid (1.1×10^{-5} M), pargyline (1.9×10^{-5} M) and test drug. After a 10 min preincubation at 37°C under an atmosphere of 95% O_2 and 5% CO_2 in a Dubnoff metabolic shaker [^{3}H]-5-HT was added in a volume of 0.1 ml yielding a final concentration of 2.6×10^{-8}M. Incubation was continued for a further 5 min and was terminated by the addition of 5 ml of ice-cold saline and by standing the beakers in ice for 10 minutes. The homogenate was separated from the medium by filtration (Millipore 24 mm diameter, pore size 0.45 μm) under vacuum. The beakers were rinsed with 5 ml of ice-cold saline and the washings filtered. The filter was then rinsed with a further 5 ml of ice-cold saline to remove any remaining radioactivity which was not associated with the tissue. The filter disc was placed in a counting vial and 15 ml of Bray's scintillant added. After 4 h at room temperature the filter disc had disintegrated and the contents of the vial were ready for counting. Incubations were carried out in the presence of four concentrations of test drug, control at 37°C and control at 0°C. The concentration of radioactivity in the incubation media was obtained by preparing incubation vessels as described above except that 0.1 ml 0.32 M sucrose was added instead of 0.1 ml homogenate and counting 1 ml of the resultant mixture. Each incubation was performed in quadruplicate. Concentrations of 5-HT taken up were calculated by dividing d min^{-1} g^{-1} of original tissue by d min^{-1} ml^{-1} of medium and corrected for diffusion by subtracting the amount taken up at 0°C.

In vivo *studies*

In vivo blockade of peripheral noradrenaline (NA) uptake was studied by investigating the effect of drug pretreatment on the ability of 6-hydroxydopamine (6-OHDA) to lower rat heart NA content. 6-OHDA (20 mg/kg) was injected intraperitoneally and rats killed 18 h later. Compounds under study were injected intraperitoneally 30 min before 6-OHDA. In experiments investigating the effect of drug pretreatment on the ability of intracerebroventricularly (i.c.v.) administered 6-OHDA to lower rat brain NA and dopamine levels, rats were anaesthetized with halothane and 6-OHDA (either 100 or 250 μg in a volume of 20 μl) was infused for 1 min into the right ventricle of the rat brain (Noble, Wurtman & Axelrod, 1967). Rats were killed 3–5 days after intraventricular injection. Compounds under study were injected intraperitoneally 30 min before 6-OHDA. Following extraction (Neff & Costa,

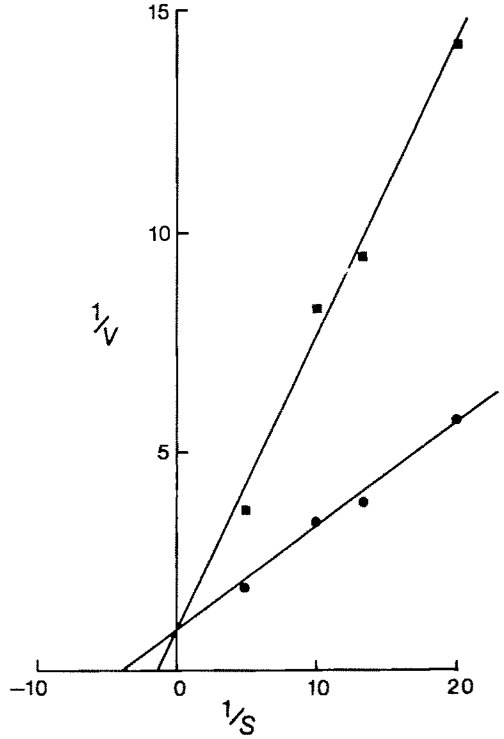

Figure 2 Lineweaver-Burk plots of (−)-metaraminol ((−)-MA) uptake by rabbit brain stem slices in the presence (■) and absence (●) of mianserin (10^{-5} M). Velocity (*V*) is taken to be net uptake of amine by slice during 30 min incubation. Substrate concentration (*S*) is µg of amine per ml of medium. Each point is the mean of at least 5 determinations. *Km* values were calculated by the method of Dixon (1953).

1966), tissue content of NA and dopamine was determined spectrophotofluorometrically (Laverty & Taylor, 1968). *In vivo* blockade of central 5-HT uptake was studied by investigating the effect of drug pretreatment on the *p*-chloroamphetamine (PCA) induced fall in rat brain 5-HT levels. Compounds were injected intraperitoneally 15 min before PCA 10 mg/kg, i.p., rats were killed 3 h after PCA injection and brain 5-HT content analysed spectrophotofluorometrically (Snyder, Axelrod & Zweig, 1965). Tissue levels of NA, dopamine and 5-HT were calculated as µg/g of wet tissue, results being corrected for 100% recovery.

The following drugs were dissolved in saline: (−)-metaraminol bitartrate, amitriptyline hydrochloride, nortriptyline hydrochloride, chlorimipramine hydrochloride, desipramine hydrochloride, *p*-chloroamphetamine hydrochloride and imipramine hydrochloride. Mianserin monohydrochloride was dissolved in distilled water. 6-Hydroxydopamine hydrobromide was freshly prepared in saline containing 5.7×10^{-3} M ascorbic acid and kept in ice. Doses refer to the free base.

Results

In vitro *studies*

Of the compounds studied for blockade of (−)-MA uptake by rabbit brain stem slices, nortriptyline and desipramine were the most potent having IC$_{50}$ values of 2.3×10^{-7} M and 4.0×10^{-7} M respectively (Table 1). Mianserin was comparable to imipramine and amitriptyline in potency. Figure 2 shows that incubation in the presence of mianserin (10^{-5} M) did not alter the V_{max} of (−)-MA uptake but did effect a change in K_m thus indicating that mianserin is a competitive inhibitor of (−)-MA uptake. The K_ms of uptake were 1.5×10^{-6} M and 4.2×10^{-6} M for control and drug-treated respectively. Kinetic experiments with desipramine (10^{-6} M) yielded similar findings, i.e. desipramine was a competitive inhibitor of (−)-MA uptake. The ability of rabbit hypothalamic minces to accumulate (−)-MA was greatly attenuated in the presence of either desipramine or mianserin at a concentration of 10^{-5} M (Table 2). In contrast, striatal (−)-MA uptake was unaltered in the presence of either desipramine (10^{-5} M) or mianserin (10^{-5} M). The accumulation of (−)-MA by both hypothalamic and striatal minces was inhibited by (+)-amphetamine (10^{-5}M).

Of the compounds studied for inhibition of [^{3}H]-5-HT uptake by rat hypothalamic synaptosomes the descending order of potency was chlorimipramine, amitriptyline, imipramine, desipramine, nortriptyline and mianserin (Table 3). It is to be noted that mianserin is approximately ten times less potent than either desipramine or nortriptyline.

Table 1 Blockade of (−)-metaraminol (MA) uptake by rabbit brain stem slices

Drug	*IC$_{50}$(M)*
Nortriptyline	2.3×10^{-7}
Desipramine	4.0×10^{-7}
Imipramine	1.9×10^{-6}
Amitriptyline	2.6×10^{-6}
Mianserin	3.0×10^{-6}

Slices were incubated for 30 min in the presence of (−)-MA (3×10^{-7} M). Drugs were added at the start of the 15 min preincubation period. IC$_{50}$ values were obtained from graphs of % inhibition of (−)-MA uptake (probability scale) plotted against log concentration of drug.

Table 2 Effects of mianserin, desipramine and (+)-amphetamine on (−)-metaraminol (MA) uptake by rabbit hypothalamic and striatal minces

	Tissue			
	Hypothalamus *Net amine uptake (μg/ml)*		*Striatum* *Net amine uptake (μg/ml)*	
Drug	*Control*	*Treated*	*Control*	*Treated*
Mianserin	0.46 ± 0.05	0.14 ± 0.02*	0.80 ± 0.05	0.78 ± 0.05
Desipramine	0.50 ± 0.04	0.08 ± 0.02*	0.54 ± 0.06	0.57 ± 0.07
(+)-Amphetamine	0.42 ± 0.05	0.14 ± 0.02*	0.63 ± 0.07	0.08 ± 0.04*

Tissues were incubated for 30 min in the presence of (−)-MA (6×10^{-7} M). Drugs (10^{-5} M) were added at start of 15 min pre-incubation period. Each result is the mean ± s.e. mean of 5–6 experiments.

* Differs from control, $P < 0.001$.

Table 3 Blockade of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) uptake by rat hypothalamic synaptosomes

Drug	*IC_{50} (M)*
Chlorimipramine	7.9×10^{-9}
Amitriptyline	3.7×10^{-8}
Imipramine	7.0×10^{-8}
Desipramine	3.1×10^{-7}
Nortriptyline	3.4×10^{-7}
Mianserin	4.0×10^{-6}

Rat hypothalamic synaptosomes were incubated for 5 min in the presence of [^{3}H]-5-HT (2.6×10^{-8} M). Drugs were added at the start of the 15 min pre-incubation period. IC_{50} values were obtained from graphs of % inhibition of [^{3}H]-5-HT uptake (probability scale) plotted against log concentration of drug.

In vivo *studies*

The ability of 6-OHDA (20 mg/kg, i.p.) to decrease heart NA levels was very sensitive to blockade by the tricyclics antidepressants (Table 4). ID_{50} values are defined as the concentration of drug required to produce a 50% block of the 6-OHDA induced fall of rat heart NA content. Mianserin was appreciably less potent than the tricyclics, having an intraperitoneal ID_{50} of 94.0 μmol/kg (28.2 mg/kg).

Three days after the intraventricular injection of 6-OHDA (100 μg) rat brain NA levels were 55.2 ± 3.4 ($n = 15$) percent of vehicle-treated controls. Pretreatment with desipramine, imipramine or chlorimipramine antagonized the ability of 6-OHDA (100 μg, i.c.v.) to lower rat brain NA levels (Table 5). The ability of 6-OHDA (100 μg, i.c.v.) to lower rat brain NA content was essentially unaltered by mianserin pretreatment (200 μmol/kg, i.p.). Doses greater than 200 μmol/kg were not studied since the intraperitoneal LD_{50} of mianserin in rats is 433.3 μmol/kg (van Riezen, 1972). The fall in brain dopamine content following 6-OHDA (250 μg, i.c.v.) administration was not significantly changed by the prior injection of either desipramine (112.8 μmol/kg, i.p.), chlorimipramine (190.5 μmol/kg, i.p.) or mianserin (200 μmol/kg, i.p.).

All the tricyclic antidepressants studied attenuated the ability of PCA (10 mg/kg, i.p.) to lower the concentration of 5-HT in the rat brain. This dose of PCA lowered brain 5-HT content to 44.5 ± 1.1% ($n = 12$) of vehicle control values at the time of death. On the other hand, mianserin, at intraperitoneal doses up to 200 μmol/kg, was essentially unable to block the PCA-induced fall in rat brain 5-HT content (Table 6).

Table 4 Blockade of the ability of 6-hydroxydopamine (6-OHDA) to lower rat heart noradrenaline (NA) levels

Drug	*ID_{50} (μmol/kg)*	*95% confidence limits (μmol/kg)*
Desipramine	3.4	2.6–4.5
Imipramine	5.7	4.3–10.4
Nortriptyline	16.7	10.3–28.9
Amitriptyline	37.9	29.6–50.9
Chlorimipramine	33.6	26.7–42.8
Mianserin	94.0	77.7–112.3

Drugs were injected i.p. 30 min before 6-OHDA (20 mg/kg, i.p.) and rats were killed 18 h after 6-OHDA administration. Heart NA levels were 20.0 ± 1.5 ($n = 15$)% of control values 18 h after 6-OHDA. The concentration of NA in the hearts of vehicle-treated control was 1.056 ± 0.023 μg/g ($n = 34$). The % inhibition of NA reduction by 6-OHDA was calculated from the formula of Carlsson, Corrodi, Fuxe & Hokfelt (1969a). ID_{50} values were obtained from % block/log dose-response curves which were constructed by the method of least squares. Each log dose-response curve had at least 3 points and each point was the mean of at least 5 determinations.

Discussion

Uptake into the presynaptic neurone is considered to be the major means by which monoamines are inactivated at the neural synapse (Iversen, 1974) and tricyclic antidepressants are thought to act by virtue of their ability to block monoamine uptake. The objective of this study was to compare the effects of mianserin, a clinically effective antidepressant, on monoamine uptake mechanisms with the effects of selected tricyclic antidepressants.

Evidence for the validity of the use of (–)-MA as an index of NA uptake *in vitro* has been presented by Giachetti & Shore (1966). The observation that secondary tricyclic antidepressants (viz. desipramine and nortriptyline) are more potent than their corresponding tertiary analogues (viz. imipramine and amitriptyline) in blocking NA uptake *in vitro* is in agreement with the findings of others (Shaskan & Snyder, 1970). The ability of mianserin to inhibit rabbit brain stem (–)-MA uptake is comparable to that of imipramine and amitriptyline and is less than that of desipramine and nortriptyline. Others have also observed an inhibition of NA uptake *in vitro* by mianserin (Baumann & Maitre, 1975; Koe, 1976; Raiteri, Angelini & Bertollini, 1976). Although

Table 5 Blockade of the ability of 6-hydroxydopamine (6-OHDA) to lower rat brain noradrenaline (NA) levels

Drug	*Dose (μmol/kg)*	*% Block (mean ± s.e. mean)*	*ID$_{50}$ (μmol/kg)*	*95% Confidence limits (μmol/kg)*
Desipramine	15.0	18.9 ± 4.3		
	30.1	41.6 ± 4.8		
	60.1	73.6 ± 5.9	34.2	29.3–41.0
Imipramine	17.9	15.0 ± 12.2		
	35.7	30.0 ± 9.5		
	71.4	73.7 ± 10.6	45.7	32.9–79.3
Chlorimipramine	47.6	24.6 ± 5.8		
	95.2	50.6 ± 5.6		
	190.5	79.7 ± 9.9	91.4	74.3–113.6
Mianserin	200.0	12.5 ± 6.3	>200.0	

Drugs were injected intraperitoneally 30 min before intraventricularly administered 6-OHDA (100 μg) and rats were killed 3 days later. Brain NA levels of vehicle-treated control rats were 0.441 ± 0.012 μg/g (n=21). For further information see legend to Table 4.

Table 6 Blockade of the ability of *p*-chloroamphetamine (PCA) to lower rat brain 5-hydroxytryptamine (5-HT) levels

Drug	*Dose (μmol/kg)*	*% Block (mean ± s.e. mean)*	*ID$_{50}$ (μmol/kg)*	*95% Confidence limits (μmol/kg)*
Chlorimipramine	11.9	25.2 ± 4.4		
	23.8	40.3 ± 6.2		
	47.6	60.6 ± 2.1	24.4	13.6–43.8
Imipramine	26.8	30.6 ± 5.2		
	53.6	48.7 ± 11.3		
	107.1	72.2 ± 10.1	52.9	32.9–89.3
Desipramine	75.2	31.8 ± 4.3		
	112.8	54.8 ± 8.5		
	150.4	63.1 ± 3.2	108.6	90.2–130.8
Mianserin	200.0	12.2 ± 8.5	>200.0	

Drugs were injected intraperitoneally 15 min before PCA (10 mg/kg, i.p.) and rats were killed 3 h after PCA administration. Brain 5-HT levels of vehicle-treated control rats were 0.535 ± 0.012 μg/g (n=28). The % inhibition of 5-HT reduction by PCA was calculated from the formula of Carlsson, Corrodi, Fuxe & Hokfelt (1969b). For remainder of legend, see Table 4.

tricyclic antidepressants are potent inhibitors of NA uptake, a characteristic feature of this group of drugs is their lack of effect on dopamine uptake (Carlsson, Fuxe, Hamberger & Lindqvist, 1966; Dorris & Shore, 1971). In contrast to (+)-amphetamine, both mianserin and desipramine had no effect on (–)-MA uptake by rabbit striatal minces. Hence the relative inability of tricyclics to block dopamine uptake *in vitro* is mimicked by mianserin. Previous studies have shown that desipramine is a competitive inhibitor of (–)-MA uptake by rabbit heart slices (Berti & Shore, 1967). This situation also holds centrally as indicated by the observation that the uptake of (–)-MA by brain stem slices was also inhibited by desipramine in a competitive manner. Like desipramine, mianserin is also a competitive blocker of brain stem (–)-MA uptake. The ability of mianserin to inhibit 5-HT uptake *in vitro* is appreciably less than that of the tricyclics studied and agrees with findings of others (Baumann & Maitre, 1975; Koe, 1976; Raiteri *et al.*, 1976).

In vivo blockade of central 5-HT uptake was quantified by studying the effect of drug pretreatment on the ability of PCA to lower rat brain 5-HT levels. In order to achieve a reduction in brain 5-HT content, PCA is actively transported into the serotoninergic neurone by the membrane amine pump and compounds blocking the pump such as the tricyclic antidepressants prevent the lowering of brain 5-HT by PCA and related analogues (Meek, Fuxe & Carlsson, 1971). In contrast to the tricyclics studied, mianserin at doses up to 200 μmol/kg was essentially devoid of effect on the ability of PCA to lower rat brain 5-HT levels and it would appear valid to conclude that mianserin has essentially no effect on central 5-HT uptake *in vivo*.

In order to lower brain tissue levels of NA and dopamine, 6-OHDA must be taken up by the neurone membrane amine pump and consequently compounds which block the pump prevent the 6-OHDA-induced depletion of NA and dopamine (Stone, Porter, Stavorski, Ludden & Totaro, 1964; Malmfors & Sachs, 1968; Evetts & Iversen, 1970; Breese & Traylor, 1972). A characteristic feature of the tricyclic antidepressants is their lack of effect on the 6-OHDA-induced fall in rat brain dopamine content (Evetts & Iversen, 1970; Breese & Traylor, 1972) and in this respect, mianserin resembles the tricyclics. In contrast to the tricyclics investigated, mianserin is essentially devoid of effect on the ability of intracerebroventricular 6-OHDA to decrease the concentration of NA in the rat brain. This result is similar to that of Leonard (1974) who showed that mianserin has no effect on the H 77/77-induced fall in rat brain NA content. However, it should be added that von Voigtlander & Losey (1976) have recently reported that mianserin rivalled imipramine in blocking the decrease in brain NA content following the intravenous injection of 6-hydroxydopa to pargyline pretreated mice. Tricyclic antidepressants are extremely potent in preventing the 6-OHDA-induced fall in rat heart NA concentration, e.g. the ID_{50} value for desipramine is 3.4 μmol/kg. Mianserin was found to be active in this model, the ID_{50} value being 94.0 μmol/kg. However, the ability of mianserin to block the depletion of rat heart NA elicited by 6-OHDA is appreciably less than that of the tricyclics studied.

Although mianserin rivals both imipramine and amitriptyline in blocking NA uptake *in vitro*, this situation clearly does not hold *in vivo*. Perhaps in the *in vivo* situation mianserin, in contrast to the tricyclics, does not readily attain and maintain a sufficiently high concentration in plasma to compete effectively with NA for attachment to the membrane amine pump. It is of interest to note that the plasma concentration of drug in patients being treated with mianserin is appreciably less than plasma levels of drug achieved in patients on tricyclic antidepressant therapy (Coppen *et al.*, 1976). Thus inhibition of NA uptake would not be anticipated in patients being treated with clinically effective doses of mianserin. This hypothesis agrees with the recent clinical report that mianserin, in contrast to amitriptyline, has no effect on the ability of tyramine to induce both a pressor response and mydriasis (Ghose, Coppen & Turner, 1976). Hence, it is improbable that the clinical effectiveness of mianserin can be attributed to inhibition of monoamine uptake. Finally, the relative lack of effect of mianserin on monoamine uptake *in vivo* offers an explanation for the ineffectiveness of mianserin in conventional screening tests for potential antidepressant agents.

Mr G. Shaw is thanked for excellent technical assistance. Thanks are extended to the following companies for gifts of drugs: Abbott Laboratories Limited (pargyline), Dista Products Limited (nortriptyline), Geigy Pharmaceuticals (chlorimipramine, desipramine and imipramine) and Merck Sharp and Dohme Limited (amitriptyline).

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(Received April 25, 1977.
Revised May 30, 1977)

Br. J. Pharmac. (1977), **61**, 315–317

THE ANTIDYSRHYTHMIC AMINOSTEROID, ORG 6001, REDUCES THE ST-SEGMENT ELEVATION PRODUCED BY CORONARY OCCLUSION IN THE DOG

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ST-segment elevation following temporary coronary artery occlusion was measured from nine epicardial leads in open-chest anaesthetized dogs. This was greatly reduced by the prior administration of the antidysrhythmic aminosteroid, ORG 6001. It is suggested that this effect is related either to a reduction in the extent and degree of myocardial ischaemia or to prevention of K^+ egress from ischaemic cells.

Introduction We have previously demonstrated that 3α-amino-5α-androstan-2β-ol-17-one (ORG 6001) administered intravenously or orally is capable of preventing the early ventricular dysrhythmias that result from coronary artery ligation in anaesthetized greyhounds (Marshall & Parratt, 1975). In addition, there was evidence that some of the metabolic indices of myocardial ischaemia (e.g. CO_2 and lactate production, K^+ efflux) were reduced in dogs treated with ORG 6001. The present study is an attempt to answer the question as to whether these beneficial netabolic effects of ORG 6001 result secondarily rom the reduction in ventricular dysrhythmias, since iese *per se* decrease myocardial perfusion (Marshall, arratt & Ledingham, 1974) or whether the drug has ome other more direct effect on the ischaemic iyocardium. We have approached this problem by ssessing the effects of ORG 6001 on the extent of myocardial injury (assessed from epicardial ST-segment changes) produced by temporary coronary occlusion in a canine model, devoid of dysrhythmic activity.

Methods Six mongrel dogs of either sex and weighing between 11–19 kg were anaesthetized with sodium thiopentone (30 mg/kg, i.v.) followed by α-chloralose 85 mg/kg. The dogs were ventilated with 100% O_2 and catheters placed in the descending aorta, pulmonary artery, right atrium and coronary sinus for pressure measurements and for blood sampling as previously described (Marshall *et al.*, 1974). After horacotomy the heart was slung in a pericardial radle and a Mersilk thread passed around the left nterior descending coronary artery (l.a.d.) about 2 cm from its origin. A triangular sheet of rubber in which were embedded nine silver electrodes (impedance 600–1200 Ω) was sutured to the anterior surface of the left ventricle so that at least six of the electrodes lay in areas supplied by the artery to be occluded (Marshall & Parratt, 1977). Care was taken to keep the epicardium moist with normal saline throughout the experiment. Aortic blood pressure, pulmonary artery pressure and epicardial electrocardiograms (ECGs) were recorded on a Mingograph ink-jet recorder.

After baseline haemodynamics, blood gases and epicardial ECGs had been obtained, the l.a.d. was occluded and epicardial ECGs at each of the nine sites were recorded by means of a rapid switching circuit (at a paper speed of 50 mm/s) at 1, 2 and 3 min after occlusion. Only short (i.e. 3 min) occlusions were used because occlusion of the artery for longer periods occasionally resulted in ventricular dysrhythmias and conduction defects; both of which mask shifts in the ST-segment. In each dog, three control occlusions were performed and occlusions were repeated 10, 30 and 60 min after injection of ORG 6001 (10 mg/kg) into the right atrium. The results were analysed statistically using the Student's *t* test for paired data.

Results Coronary artery occlusion resulted in a time-related elevation of the ST-segment in the epicardial leads overlying the ischaemic area (Figure 1); the ST-segment in leads lying outside the affected area remained unchanged. Heart rate and pulmonary artery pressure remained unchanged throughout occlusions but there was always a small (5–15 mmHg) fall in aortic blood pressure. As previously reported (Wendt, Canavan & Michalak, 1974), there was evidence that the first occlusion resulted in more marked ST-changes than subsequent occlusions. However, these subsequent occlusions (up to six) produced similar changes in the ST-segment elevation in control dogs.

The total ST-segment elevation (assessed at 3 min) in five control, untreated dogs was 34 ± 4 mV (first occlusion), 26 ± 3 mV (second occlusion), 25 ± 4 mV (third occlusion), 31 ± 4 mV (fourth occlusion) and 36 ± 2 mV (fifth occlusion). There was no significant difference in control dogs between the second, third, fourth or fifth successive occlusions.

Administration of ORG 6001 (10 mg/kg) caused similar transient haemodynamic changes to those

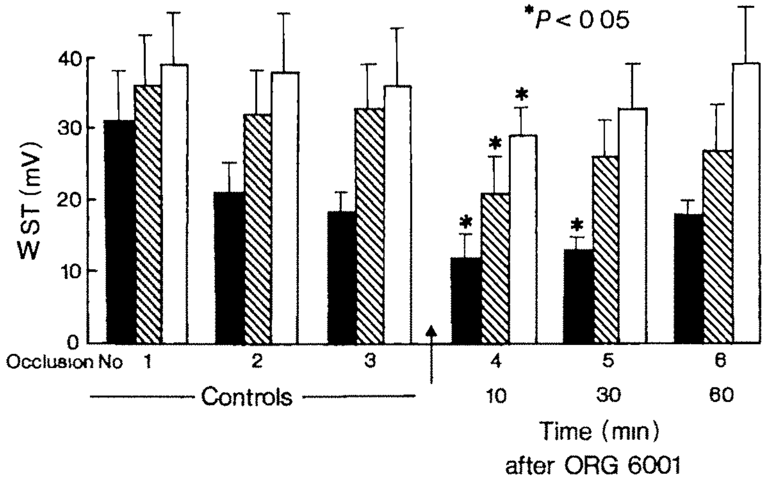

Figure 1 Total ST-segment elevation produced at 1 min (solid columns), 2 min (hatched columns) and 3 min (open columns) after acute occlusion of the left anterior descending coronary artery in anaesthetized dogs. At the arrow ORG 6001 (10 mg/kg) was administered intravenously. Histograms and bars represent the means and standard errors respectively of six observations.

already reported (Marshall & Parratt, 1975) and all parameters had returned to normal within 5 min of the injection. At this dose, ORG 6001 significantly reduced ST-segment elevation throughout the occlusion (Figure 1) and in addition reduced the mean number of leads showing >2 mV elevation (Maroko, Kjekshus, Sobel, Watanabe, Covell, Ross & Braunwald, 1971) from 6.6 ± 0.3 to 5.1 ± 0.2 ($P < 0.05$). These effects of ORG 6001 were relatively short-lasting and ST-elevation induced by occlusion returned towards control values 30 to 60 min after drug administration (Figure 1).

Discussion Although the mechanisms underlying ST-segment changes are still incompletely understood, many experimental studies have demonstrated that epicardial ECG mapping can be used to assess the degree and extent of myocardial ischaemia. Changes in the ST-segment correlate well with the subsequent depletion of CPK (Maroko *et al.*, 1971), with the degree of change in coronary blood flow and with the extent of anaerobic metabolism (Karlsson, Templeton & Willerson, 1973).

This present study has shown that an aminosteroid antidysrhythmic drug, ORG 6001, is capable of reducing ST-segment elevation produced by short coronary occlusions. Previous studies (Maroko *et al.*, 1971; Smith, Singh, Nisbet & Norris, 1975; Wendt *et al.*, 1975) have demonstrated that β-adrenoceptor antagonists and verapamil, which decrease myocardial oxygen requirements, decrease ST elevation produced by coronary artery occlusion. Clearly ORG 6001 does not share this mechanism of action since the drug is devoid of β-blocking activity and does not reduce myocardial oxygen consumption (Marshall & Parratt, 1975).

There are two other possible explanations for this decrease in ST-elevation. Firstly, at the dose used in this study, ORG 6001 prevents K^+ efflux from ischaemic myocardial cells into coronary venous blood (Marshall & Parratt, 1975). Potassium efflux *per se* would be expected to elevate the ST-segment (Fozzard & DasGupta, 1976) and indeed we have unpublished data which show a good relationship between extracellular K^+ level and ST-segment elevation. A second and possibly related mechanism concerns the electrical events causing ST-segment elevation. Since ST elevation is a reflection of the injury potential generated *between* normally perfused and ischaemic tissue (Cohen & Kaufman, 1975) any agent which normalizes action potential characteristics in these areas would be expected to decrease ST-segment elevation. Of interest are findings (Salako, Vaughan Williams & Wittig, 1976) that ORG 6001 shortens the action potentials in rabbit ventricular conducting system so that duration is uniform along this system.

The ability of ORG 6001 to reduce ST-segment elevation may have clinical relevance to its antidysrhythmic properties since a good correlation has been shown between infarct size and the incidence of ventricular ectopics in patients with recent myocardial infarcts (Roberts, Husain, Ambos, Oliver, Cox & Sobel, 1975). It would be of considerable interest to investigate whether other antidysrhythmic agents which prevent K^+ efflux are also capable of decreasing ST elevation produced by experimental coronary occlusion.

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(Received June 28, 1977)

Br. J. Pharmac. (1977), **61**, 318–320

REDUCED ADRENAL AMINE SYNTHESIS IN SPONTANEOUSLY HYPERTENSIVE RATS AFTER LONG-TERM TREATMENT WITH PROPRANOLOL

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Male spontaneously hypertensive rats were either fed a diet containing 3 mg/g of (±)-propranolol, giving a mean daily intake of 178 ± 4.2 mg/kg, or a control diet from 7 weeks of age for 6 months. Three days after cessation of propranolol-treatment, the arterial blood pressure of the treated group was about 10% lower than that of the control group. Adrenal dopamine content and tyrosine hydroxylase activity were reduced to 76% and 71% of the control values, respectively. The results indicate that propranolol causes a reduced sympathetic discharge through its central action, although blockade of *β*-adrenoceptor-mediated local regulatory mechanisms cannot be excluded.

Introduction The mechanism of the antihypertensive action of *β*-adrenoceptor-blocking drugs is still a matter of debate. Several investigations point to an involvement of the central nervous system. A hypotensive response has been recorded after intracerebroventricular administration of propranolol to conscious cats (Day & Roach, 1974) or conscious rabbits (Reid, Lewis, Myers & Dollery, 1974; Myers, Lewis, Reid & Dollery, 1975). Reduced splanchnic discharge after intravenous administration of propranolol to conscious rabbits has also been reported (Lewis & Häeusler, 1975).

The present study was undertaken to investigate certain peripheral effects pointing to decreased sympathetic activity following long-term treatment of spontaneously hypertensive rats of the Okamoto strain (SHR) with propranolol.

Methods Male SHR were treated from 7 weeks of age, for a period of 6 months, with (±)-propranolol administered in pelleted food, an ordinary rat diet with an admixture of (±)-propranolol 3 mg/gram. The daily intake of the drug averaged 178 ± 4.2 mg/kg with a somewhat lower value towards the end of the study. Control SHR of the same age received a similar diet without propranolol, and were otherwise treated identically.

At the end of the six months' treatment period the propranolol diet was replaced with the control diet. Under pentobarbitone (75 mg/kg i.v.) anaesthesia, a polyethylene catheter was inserted into one carotid artery and passed out through the skin of the neck for blood pressure recording in the unrestrained animal in the morning of the third day after cessation of the drug; the animals were killed and the adrenals immediately taken out, weighed and frozen on dry ice for later analysis. The adrenals of one side (alternating) were used for analysis of noradrenaline, adrenaline and dopamine (Bertler, Carlsson & Rosengren, 1958; Atack & Magnusson, 1970; Atack, 1973) and the other for *in vitro* determination of tyrosine hydroxylase activity (TH), according to Nagatsu, Levitt & Udenfriend (1964).

Results The weight of rats in the treated group was slightly lower both at the start of the study (185 ± 5.8 g, $n = 10$) and at the end (299 ± 7.7 g, $n = 10$) compared with the control group (219 ± 3.7 g, $n = 9$ and 344 ± 9.4 g, $n = 9$, respectively). However, there was no difference in growth rate (i.e. percentage increase per week) between the two groups. Thus, propranolol did not seem to retard growth in the treated group.

The parameters studied are presented in Table 1. Blood pressure was approximately 10% lower in the treated group ($P < 0.05$). This antihypertensive effect was paralleled by a reduction in adrenal dopamine (76% of control value, $P < 0.01$). Neither the weight of the adrenals nor their content of noradrenaline or adrenaline was significantly affected. No apparent behavioural changes (i.e. in motility, irritability, aggressiveness) were observed in the treated group compared with controls.

Discussion The lower blood pressure of propranolol-treated SHR was accompanied by a reduction in adrenal dopamine content and TH activity, reflecting lowered catecholamine synthesis in adrenal medullary cells (see e.g. Mueller, Thoenen & Axelrod, 1969; Snider & Carlsson, 1972). A similar effect was recently described by Chubb & Raine (1976) in sympathetic ganglia of rabbits after 3–6 days treated with (±)-propranolol, 4 mg/kg subcutaneously twice daily. The most obvious explanation for lowered catecholamine synthetic activity of adrenal medullary or sympathetic ganglion cells would be reduced impulse frequency in the sympathetic system (see e.g.

Thoenen, Mueller & Axelrod, 1969; Carlsson & Lindqvist, 1974). Such an interpretation of the data is supported by the finding of Lewis & Häeusler (1975) of reduced splanchnic discharge in the conscious rabbit after intravenous propranolol administration.

It is unlikely that the results of the present study are due to the membrane-stabilizing action of propranolol, since preliminary data from this laboratory indicate that the β_1-selective antagonist metoprolol, which is devoid of membrane stabilizing properties, also reduces adrenal dopamine and TH activity in SHR after long-term oral treatment in doses which lower blood pressure. The involvement of β-adrenoceptor-mediated local regulatory mechanisms in sympathetic neurones and adrenal medullary cells cannot be excluded at present. A presynaptic β-adrenoceptor, mediating a weak positive feed-back regulation of noradrenaline release from sympathetic nerve terminals has been described (see e.g. Dahlöf, Åblad, Borg, Ek & Waldeck, 1975). Theoretically, blockade of this adrenoceptor should result in a reduced rate of amine synthesis. In addition, a weak β-adrenoceptor-mediated induction of TH in rat superior cervical ganglia has been postulated (Hanbauer & Costa, 1975). However, as these local regulatory mechanisms seem to be of a low potential it is questionable if they suffice to explain the present results. A centrally-mediated reduction of the sympathetic outflow seems at present to be a more probable explanation for the decreased catecholamine synthetic activity of the adrenal medulla after long-term propranolol treatment and the same mechanism may, at least in part, contribute to the antihypertensive action of β-adrenoceptor blocking drugs.

This study was supported by the Swedish Medical Research Council (project No 2862 and 3884) and AB Hässle, Mölndal.

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Table 1 Mean arterial blood pressure, heart rate, adrenal weight and noradrenaline (NA), adrenaline (Ad), dopamine and tyrosine hydroxylase activity (TH) 3 days after cessation of chronic propranolol treatment (6 months) of spontaneously hypertensive rats

	Control (n=9)	*Propranolol (n=10)*	*Significance (Mann-Whitney)*
Mean arterial blood pressure (mmHg)	160 ± 4.3	146 ± 5.3	$P<0.05$
Heart rate (beats/min)	386 ± 16	348 ± 16	NS
Adrenal weight (mg/gland)	27 ± 1.1 (*n*=18)	25 ± 0.5 (*n*=20)	NS
Adrenal NA (µg/gland)	5.97 ± 0.517	5.42 ± 0.421	NS
Adrenal Ad (µg/gland)	13.58 ± 1.139	13.35 ± 0.793	NS
Adrenal dopamine (ng/gland)	464 ± 36.3	352 ± 16.8	$P<0.01$
Adrenal TH (nmol/gland)	19.54 ± 1.926	13.81 ± 1.007	$P<0.01$

The Mann-Whitney U-test was used for statistical comparisons.

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(Received July 19, 1977)

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Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge